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19 ANNUAL REPORT

DIVISION OF CANCER AND PREVENTION

NATIONAL CANCER INSTITUTE

October 1, 1980 through September 30, 1981

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ANNUAL REPORT

DIVISION OF CANCER CAUSE AND PREVENTION

Richard H. Adamson, Ph.D., Acting Director

October 1, 1980 through September 30, 1981

OVERVIEW

Three years ago, the Division of Cancer Cause and Prevention (DCCP) was reorganized according to a plan which clearly separated the intramural and extramural programs. Since that time the intramural research program has been undergoing changes and in July 1981, four new laboratories were created in order to strengthen the areas of chemical carcinogenesis, tumor promotion and studies of transformation at the molecular level. These changes will be reflected in next year's annual report. The DCCP organizational chart prior to these changes is shown in Figure 1. Also, an effort has been made to bring about better interactions between the biological carcinogenesis and chemical carcinogenesis laboratories and to have these labortories interact with the three branches in epidemiology. The distribution of funds for the intramural laboratories was 17.2% and that for Field Studies and Statistics (Epidemiology) was 14.9% of the total DCCP budget.

The extramural component, known collectively as the Carcinogenesis Extramural Program, comprises several major activities: Biological Carcinogenesis, Chemical and Physical Carcinogenesis, and Epidemiology, including major aspects of the Smoking and Health and the Diet and Nutrition Programs. The overall budget for research in these areas is given in Table 1 and Figure 2.

This year the Division completed the movement of the Laboratory of Viral Carcinogenesis to the government-owned facilities at the Frederick Cancer Research Center (FCRC), Frederick, Maryland, and in addition, moved the Laboratory of Experimental Pathology and a new laboratory, the Laboratory of Comparative Carcinogenesis, to the FCRC. During the next year, the Division proposes to relocate the Laboratory of Cellular and Molecular Biology to Building 37 and to move the Laboratory of Chemoprevention to Building 41. These moves and the creation of the new laboratories, as well as future changes, will return all remaining off-reservation DCCP laboratories to Government facilities; provide geographic unity of individual laboratories; strengthen the area of chemical carcinogenesis; help integrate biological, chemical and physical carcinogenesis programs and bring about interactions with the Field Studies and Statistics Program; and reduce funds needed for resource support laboratories.

The past year has seen a continued reduction in overall contract support. This has been realized by gradual phase-out of contract-supported, investigator-initiated reseach in areas where grants provide adequate coverage; by reducing activities which provide materials and services; and by initiating various cost-recovery mechanisms. By the same token, as support for research contracts has dropped, support for investigator-initiated research grants has continued to increase.

In addition to its intramural and extramural research, the Division has been involved in other activities during the past year which merit attention. These are:

1. Frederick Cancer Research Center (FCRC)

   Research conducted by the contractor began in the early 1970s as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRC has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens. As a separate effort, the contractor provides support services and materials to intramural investigators who work in NCI laboratories at the Frederick facility. Government and contractor scientists have combined their efforts to create a center of excellence for cancer research.

2. The DCCP Board of Scientific Counselors

   Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, epidemiology, immunology, pathology, genetics, and cell biology, the members of the Board advise the Division Director on a wide variety of matters which concern the progress of the Division's programs.

   One particularly important responsibility of the Board has been the examination of the productivity and performance of staff scientists by site visit review of the intramural laboratories. These visits have been conducted by teams which, as a rule, comprise two to three members of the Board and four or more investigators from the scientific community outside NIH whose special fields of expertise match those of the scientists in the Laboratory of Branch being reviewed. The site visit reports, which reflect a consensus of the members of the team, are critically examined by the entire Board of Scientific Counselors and, after dicusssion, recommendations based on the reports are submitted to the Division Director.

   The first cycle of site visits to the Division's entire intramural operation was completed last year with the exception of the site visit to the Laboratory of Viral Carcinogenesis, which will take place on September 14-15, 1981. It is anticipated that the next cycle of site visits will begin in one to two years.

   Another important function of the Board is that of concept review, in which the members pass judgment on concepts for grant- or contract-supported activities. The Board continued to examine new concepts this year as new directions in extramural research activities developed.

   Several workshops involving Board members, as well as participants from the scientific community outside NIH, were held this year. As a consequence, new initiatives resulted using the mechanism of a Request for Grant Application (RFA) in the areas of interspecies studies of chemical carcinogenesis, tumor promotion, epidemiology and smoking and health.

Continuing modifications of funding mechanisms approved by the Board include: a gradual transfer of current resources to a cost-reimbursement system, increased availability of funding to support new resource activities, the phasing out of contract-supported research in areas adequately covered by grant applications and an increased use of RFAs to stimulate research activity in high priority areas.

The Division is most grateful to the members of the Board for giving so generously of their time, effort, and expertise in helping to guide the future of the Division's programs. There is every expectation that the Board will continue to play a vital role as the Division's foremost advisory group.

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Figure 1

DCCP ORGANIZATIONAL CHART

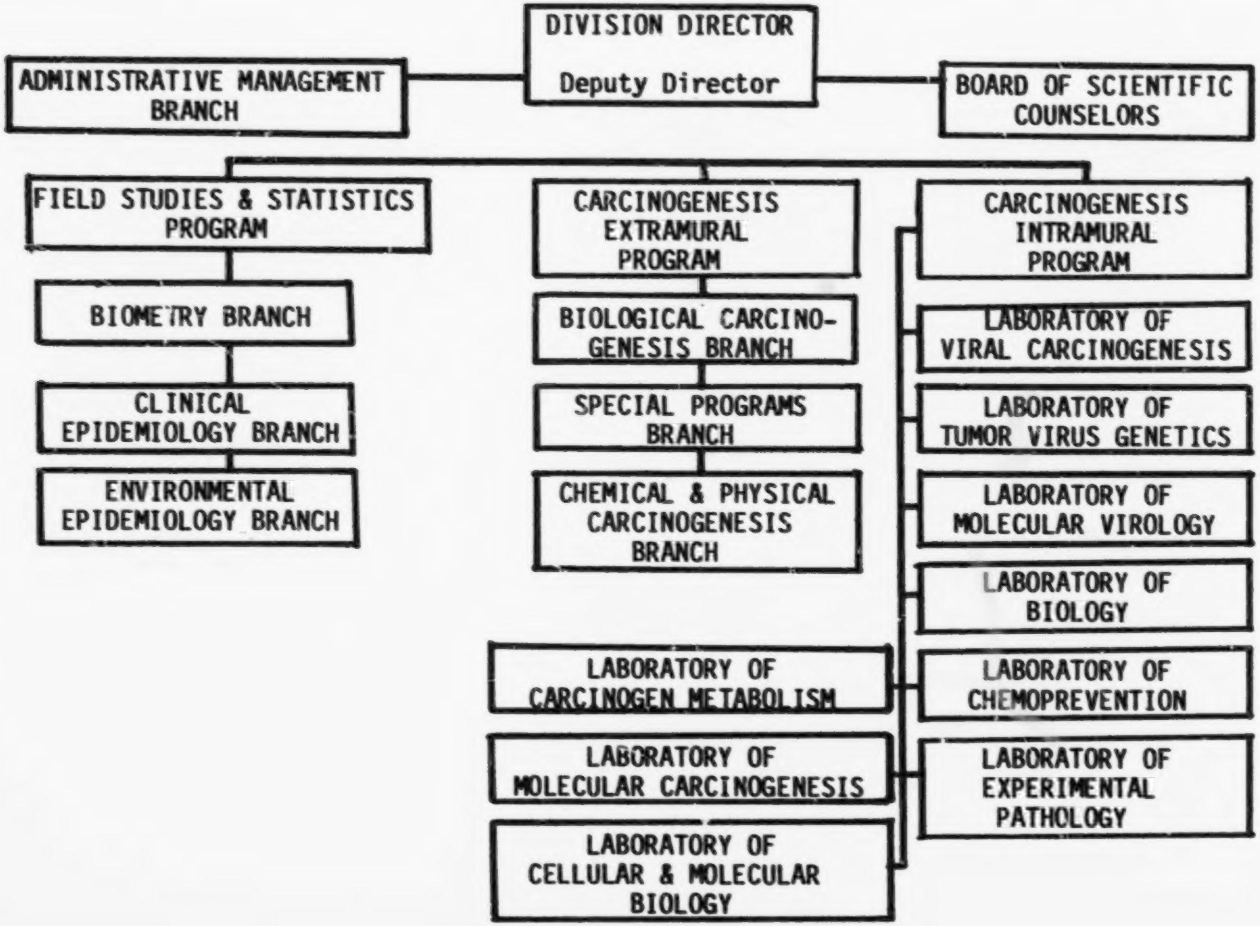
DIVISION DIRECTOR
Deputy Director
ADMINISTRATIVE MANAGEMENT BRANCH
BOARD OF SCIENTIFIC COUNSELORS
FIELD STUDIES & STATISTICS PROGRAM
BIOMETRY BRANCH
CLINICAL EPIDEMIOLOGY BRANCH
ENVIRONMENTAL EPIDEMIOLOGY BRANCH
CARCINOGENESIS EXTRAMURAL PROGRAM
BIOLOGICAL CARCINOGENESIS BRANCH
SPECIAL PROGRAMS BRANCH
CHEMICAL & PHYSICAL CARCINOGENESIS BRANCH
CARCINOGENESIS INTRAMURAL PROGRAM
LABORATORY OF VIRAL CARCINOGENESIS
LABORATORY OF TUMOR VIRUS GENETICS
LABORATORY OF MOLECULAR VIROLOGY
LABORATORY OF BIOLOGY
LABORATORY OF CHEMOPREVENTION
LABORATORY OF EXPERIMENTAL PATHOLOGY
LABORATORY OF CARCINOGEN METABOLISM
LABORATORY OF MOLECULAR CARCINOGENESIS
LABORATORY OF CELLULAR & MOLECULAR BIOLOGY

Table 1

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER CAUSE AND PREVENTION

Table of Mechanisms by Organizational United Based on Estimated Current Level of Expenditures

(dollars in thousands)

September 30, 1981

	Office of the Division Director	Carcinogenesis Intramural	Carcinogenesis Extramural	Field Studies and Statistics	Total
Intramural	2,127	27,201	1,630	7,796	38,754
Contracts	8,800	9,504	26,669	23,630	71,603
CREG/RFA	---	---	2,740	351	3,091
Preventive Oncology	---	---	574	---	574
Research Project Grants	---	---	99,369	---	99,369
Total	10,927	36,705	133,982	31,777	213,391

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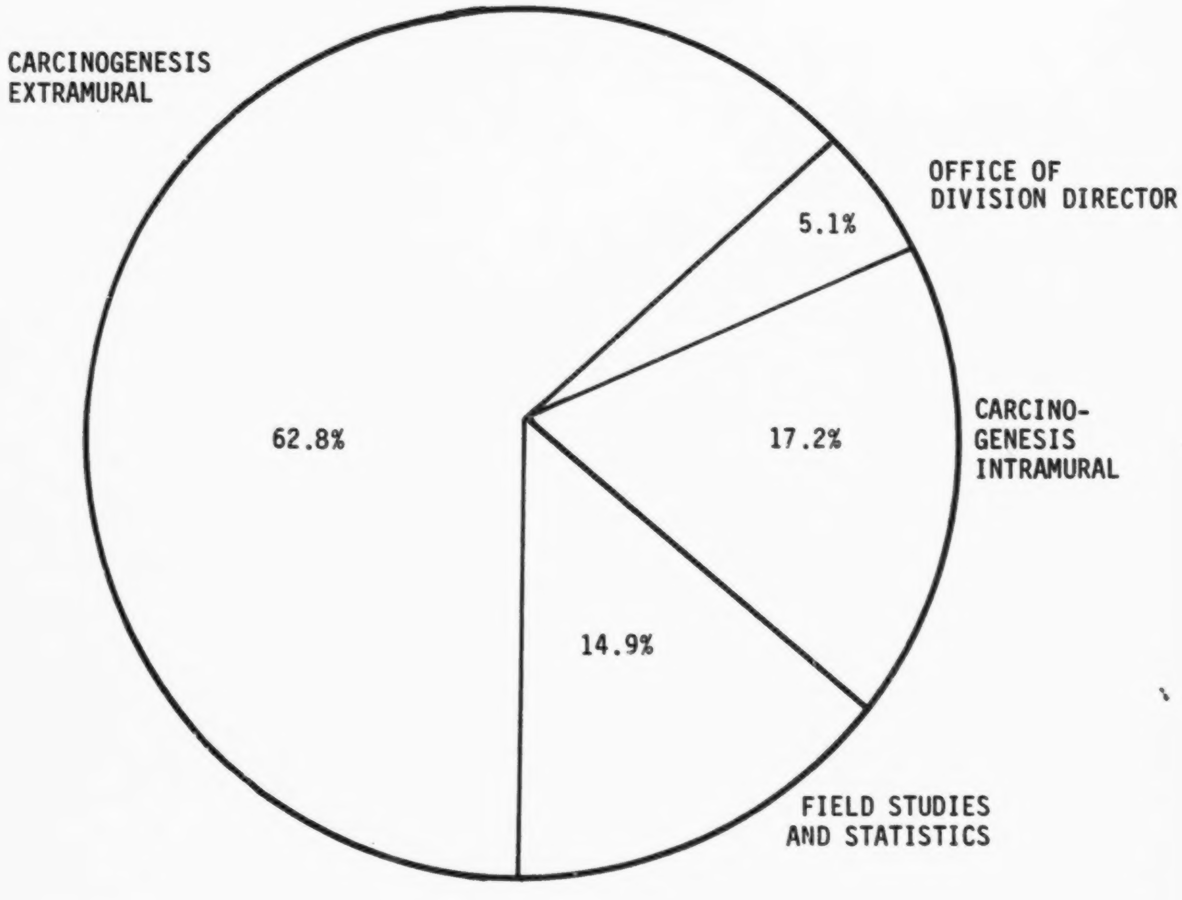
Figure 2

NATIONAL CANCER INSTITUTE

Division of Cancer Cause and Prevention

Current Distribution of Funds

Fiscal Year 1981



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SCIENTIFIC HIGHLIGHTS

Introduction: The Division of Cancer Cause and Prevention is responsible for planning and conducting the Institute's program of coordinated research on cancer causation and prevention. The Division supports both intramural laboratories and branches and extramural programs which seek to elucide the mechanisms of cancer induction at each step of the cellular process from initiation to transformation of normal cells to malignant cells. The overall purpose of these studies is to provide information for reversing, interrupting or preventing this process before the development of clinical disease.

To accomplish these goals, investigators pursue fundamental studies on normal and malignant cells and on carcinogens or procarcinogens, such as viruses and chemicals, using the disciplines of cellular and molecular biology, immunology, biochemistry, and microbiology and epidemiologic studies of human populations to identify risk factors predisposing to various cancers, using the disciplines of clinical medicine, genetics, mathematics, and biometrics.

Excellent model systems are available to scientists studying the effects of exposure to a diversity of potentially carcinogenic factors in the environment. Much fundamental information has been obtained studying tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Investigations in the area of biological carcinogenesis have shown that viral information present as nucleic acid sequences in the genes of normal cells and replicating with them may be intimately involved in the development of cancer. Triggered by chemical carcinogens, radiation, hormones, aging factors and other influences, these highly conserved viral sequences may direct the synthesis of proteins responsible for malignant transformation of the cell. The work is conducted on animal and human cancers under several general categories, including virus-cell interactions, virus-host interactions, and molecular pathways of viral replication.

Similarly, chemical carcinogens, both synthetic and naturally occurring, provide a diverse group of chemicals with cellular and tissue selectivities that influence transformation and progression from the normal to the malignant state. Studies in the area of chemical/physical carcinogenesis cover a broad range of approaches with emphasis on the mechanisms of action of chemical and physical agents. Investigators have focused on the effects of carcinogens on cell structure and function, the relationships between molecular structure and carcinogenic activity, enzyme systems associated with the metabolic activation of procarcinogens to carcinogenic forms, the effects of the binding of carcinogens to DNA, and systems that repair DNA damage. Other relevant efforts include investigation of the role of various factors in the environment of the cell; for example, promoters, hormones and growth factors which may be required for the progression of the "initiated" cell to the malignant state.

Finally, studies on the natural history of cancer in humans and on the incidence of cancers in different geographic locations help to identify causal associations of various intrinsic and extrinsic risk factors with various cancers. In view of the increasing importance of nutrition and lifestyle in the causation or prevention of cancer, in particular the role of vitamins and trace elements in the diet, alcohol consumption and smoking, special emphasis has been given to projects that may have more immediate health implications. Many studies deal with determining mutagens/carcinogens in foods, natural inhibitors in foods, or assessing the carcinogenic components in cigarettes, and the influences of the total smoking experience. In addition, major studies on cancer incidence, mortality and survival in the U.S., cancer incidence in the workplace, effects of low-level radiation, environmental pollutants in air, water, and soil are under investigation.

MAJOR FINDINGS:

Biological Carcinogenesis

Advances in recombinant DNA and nucleotide sequencing technologies have been applied to defining the structure and function of RNA tumor viruses, cell-derived transforming genes and gene products. The actions of these genes and gene products initiate and maintain the neoplastic state in cells infected by certain tumor viruses. The data suggest that: avian and mammalian genomes contain nucleotide sequences related to various retroviral sequences; some sequences emerged early in evolution; and most sequences have been conserved during the period of evolution.

This year, many studies report the detection, molecular cloning, and genomic organization of retroviruses. The proviral genome of Abelson murine leukemia virus (A-MuLV), a transforming retrovirus, was molecularly cloned. A DNA fragment containing the viral genome was shown to transform NIH/3T3 cells at high efficiency. Transfection with subgenomic A-MuLV DNA clones further localized the region necessary for transformation. Similarly, the integrated form of simian sarcoma virus, the only known transforming retrovirus from a primate, was cloned. A specific sequence was uniquely represented within the normal cellular DNA of several unrelated mammalian species, including man. This gene shows close sequence homology to woolly monkey cellular DNA and may be a recombinant of a type C helper virus and woolly monkey cellular gene. Chimpanzee DNA was found to contain a single family of sequences related to other primate retroviruses. These sequences, obtained from the closest living relative of man, could be expected to provide the best DNA hybridization probes for the detection of related endogenous viral sequences in the DNA of man. Such probes easily detected related sequences in cellular DNA from gorilla, baboon, rhesus, Colobus monkeys and even mice but did not detect them in man, gibbon or orangutan.

Virologists have begun to understand how some human viruses convert normal cells to malignant ones. One of the four genes in the Rous sarcoma virus (RSV) carries all of the information necessary for the initiation of transformed phenotype in cells infected with this virus. Studies of the gene product, pp60src, have demonstrated that it is a kinase. Cells transformed by the Rous sarcoma virus contain levels of phosphotyrosine in proteins which are up to 50 fold greater than the levels present in uninfected cells. These results indicate that modification of one or more cellular polypeptides catalyzed by pp60src is crucial for cellular transformation by RSV. Since RSV transformed cells contain cellular proteins which are newly phosphorylated on tyrosine residues, several lines of genetic evidence indicate that the protein which modified these cellular proteins is pp60src. At least eight cellular substrates for pp60src appear to exist in the RSV system. One of these, vinculin, is a polypeptide located in membrane structures called adhesion plaques which seem to play a role in the adherence of cells to surfaces.

Two segments of the src gene of Harvey sarcoma virus have been identified in normal rat DNA. One is colinear with the src gene of Harvey virus, while the other has untranscribed intervening pieces. Each normal rat gene is capable of inducing malignant transformation of cells and encodes for P21, the src gene product of Harvey virus. The results prove unambiguously that activation of a normal cellular gene can cause malignant transformation. The p21 coded by the viral and cellular src genes show interesting differences that may help to elucidate biochemical properties associated with the transformation event and represent a family of divergent genes within rat DNA.

Other studies have been concerned with elucidation of the regulatory signals which are responsible for gene expression. Studies using the murine sarcoma virus model have provided the first insight as to how a normal cell's DNA sequence may be activated to transform the cell. For example, it was recently found that specific viral promoter regions exist at the end of proviral DNA. These regions, known as long terminal repeats (LTR), can profoundly enhance the promotion of cellular genes. The LTR appears to have transcription activation properties and may be a general mechanism for oncogenesis.

A cloned subgenomic fraction of Moloney sarcoma virus (MSV) containing the acquired cell sequence v-*mos* was able to transform cells at low efficiency in DNA transfection assays. When v-*mos* was covalently linked to MSV sequences containing LTR, the transforming efficiency was enhanced 1,000-fold. Similarly, a normal mouse cell fragment containing sequences homologous to v-*mos*, namely c-*mos*, was identified and isolated. The c-*mos* containing fragment was unable to transform cells. Cells transformed by v-*mos* or c-*mos*, with LTR elements positioned either upstream or downstream to the transforming sequence, express RNA in the same configuration. The LTR element appears to activate RNA expression of the *mos* sequence when it is positioned either before or after this sequence. The LTR also appears to have transcription activation properties in addition to providing signals for either starting or polyadenylation of RNA transcripts.

In parallel studies on Harvey and Kirsten sarcoma, integrated proviruses contain promoters of transcription of src genes which are found within the long, terminal repeats, LTR. Promoters for RNA polymerase II and surprisingly, RNA polymerase III have been identified. No mRNA with protein coding potential has previously been associated with RNA polymerase III activity. Since activation of cellular src genes can induce transformation, transcriptional control studies may elucidate natural control mechanisms and targets for activation of endogenous src genes by chemical and physical carcinogens.

Similarly, the nucleotide sequence at the host cell-proviral junction of MuMTV was determined using the C3H strain of virus. Proviruses cloned from rat cells infected with MuMTV, a type B retrovirus regulated by glucocorticoid hormones, show the structural features of transposable elements: short inverted repeats conclude long direct repeats at the ends of viral DNA, and short sequences of cellular DNA are duplicated during integration and flank each provirus. Thus, during integration a precise site in viral DNA joins to non-homologous sites in host cell DNA. A full length DNA from an endogenous MMTV (C$_3$Hf mouse) has also been molecularly cloned and retains glucocorticoid regulatory signals. The molecular clone has been covalently linked to p21 src gene of Harvey virus and LTR of MMTV; these molecules are hormonally responsive to glucocorticoids. This finding has fundamental significance in understanding the role of hormones in the transformation process.

The study of hormone-line control factors that have profound influence on the genetic and phenotypic expression of cells is another promising area of research. A family of transforming growth factors (TFGs) related to, but distinct from, epidermal growth factors (EGF) has been partially purified from the conditioned medium of human tumor cells as well as from extracts of the tumor cells themselves. These biologically active peptides produce at least some of their effects by interacting with EGF-specific membrane receptors. This action can be blocked by very small amounts of retinoids (vitamin A derivatives) and another class of inhibitors, pseudodipeptides.

Human TGFs were found to be closely associated with tyrosine-specific protein kinase activity which has been strongly associated with transformed cells. A human tumor line with the greatest concentration of available EGF receptors exhibited a pronounced increase in total phosphotyrosine in response either to mouse EGF or to TGF. The overall extent of tyrosine phosphorylation in these growth factor-treated cells was comparable to those characteristic of RNA tumor virus-transformed cells.

This year, sarcoma growth factors (SGF) were further characterized. Three major peaks of transforming peptides, SGFs, were found in serum-free supernatant fluids from virus-transformed mouse 3T3 cells. The first consisted of a heterogeneous group of proteins with apparent molecular weights around 20,000; the second contained anchorage-independent growth-stimulating activity with an apparent molecular weight of 9,000-10,000; and the third contained transforming activity corresponding to EGF-competing activity in the 6,000 molecular weight range. SGFs produce profound phenotypic alteration in cultured cells and confer on them the ability to behave as non-permanently transformed cells.

A new group of immunosuppressive factors (ISFs) were found in crude supernatant preparations of Moloney murine sarcoma virus-transformed mouse 3T3 cells; normal mouse 3T3 cells release little or none. The major activity is an 8,000 molecular weight peptide. Tumor cells may be able to produce two families of peptides that protect their ability to proliferate and spread in the body; the first, SGF and TGF, stimulate the growth of tumor cells; the second, ISF, is a potent inhibitor of the clonal expansion of T cells and the development of cytotoxic lymphocytes.

The murine mammary tumor viruses (MMTV) are unique in being the only viruses known to induce mammary carcinomas. Several different mouse mammary tumor virus MMTV variants may be involved in the malignant transformation of epithelial cells. Strains of mice with a low incidence of mammary tumors may harbor several endogenous proviruses. Spontaneous mammary tumors may arise without amplified MMTV proviral information or as the result of preferential expression of a single endogenous provirus.

Studies continue to report the finding in human mammary cancer of an antigen which is immunologically related to the major glycoprotein (gp52) of MMTV and to determine the significance of its cross-reactivity. Significant progress has been made in the purification of the similar, related antigen from human breast cancer cell lines.

Monoclonal antibodies, both of human and mouse origin, have been generated against human mammary carcinoma cells. The antibodies were reactive to cell extracts of carcinoma cells but negative against similar extracts from a variety of normal and malignant control cells. The range of reactivity of these antibodies is being evaluated by a number of sensitive methods.

A number of studies focus on the role of T-antigens in the simian virus 40/polyoma system (SV 40/polyoma) which may be critical in DNA replication, transcriptional regulation and cell transformation by these viruses. Many host-range mutants of this virus system have become valuable for studying and understanding the cellular changes related to transformation by these viruses. Apparently, small and middle T-antigens are required for the expression of transformation, while large T-antigen promotes transformation efficiency.

In continuing studies of the association of DNA viruses with human malignancy, the Epstein-Barr virus (EBV) directed nuclear antigen (EBNA) has been prepared from Raji cells to a purity of 90-95 percent. Peptide mapping and cleavage studies have shown that the antigenically active 48K component, EBNA, has no peptides in common with the cellular proteins that co-purify with it in the early phases of purification nor with degradation products of the transformation-related 53K protein.

In a study of very late relapses in Burkitt's lymphoma (BL) patients, it was observed that 10 percent of the patients may relapse after remission periods of one to six years (the duration of study). These patients maintained elevated early antigen-restricted type (EA-R) antibody titers throughout their remission period, indicating that they were not out of danger as was subsequently confirmed by the later relapses. Whether the relapses are due to surviving BL cells or to new tumor induction is not known. In the absence of detectable tumors, the elevated EA-R titers may be attributed to a highly active persistent EBV infection.

EBV serological markers for diagnosis and prognosis of patients with nasopharyngeal carcinoma indicate that antibody titers to EBV-induced membrane antigen (MA) measured by the antibody-dependent cellular cytotoxicity (ADCC) assay has probable prognostic value. The data show that high anti-MA ADCC titers at diagnosis were indicators of a good prognosis following therap, whereas low titers were indicators of poor prognosis. The results also suggest that antibody to MA may function actively *in vivo* against these tumors.

CHEMICAL CARCINOGENESIS

This year studies on the mechanisms of chemical carcinogenesis have received increased programmatic emphasis. Scientists are working on the hypothesis that chemical carcinogenesis is a complex process with distinct phases, progressing from a long latency period to multiple stages of preneoplastic to neoplastic change. In the animal host, many systems may be required to activate a chemical or its carcinogenic form; any minor modification in chemical structure could lead to profound changes in a metabolic pathway. Current evidence indicates that the carcinogenic insult may be an early event involving a structural change in the DNA of certain cells -- a point mutation or perhaps more generalized damage to DNA -- as a precursor to transformation to a cancer cell. Significant interactions may occur epigenetically in the cytoplasm or at the cell surface. Nevertheless, it is possible that the carcinogen-bound or damaged DNA can be repaired and that failure or inability to repair this damage may be linked to cancer. The resulting increased expression of a gene or increased transcription of a gene product which in some way regulates or controls cell growth and differentiation may be responsible for maintaining the transformed state. Finally, there may be other factors required for transformation to malignancy. For example, tumor promoters, hormones or other growth factors may enhance or accelerate the expression of neoplastic potential in the latent or initiated cell. Understanding the mode of action of these promoters is an important goal in carcinogenesis research.

Currently, laboratory investigations seek to understand how exogenous carcinogens and endogenous factors are processed by enzymatic mechanisms. Cytochrome P-450 mixed function oxidases have a range of substrate specificities that might account for the activation and detoxification of many chemical carcinogens. Recombinants carrying methylcholanthrene (MC)-type cytochrome P-450 sequences have been used to demonstrate that microsomal cytochrome P-450 is elevated four-fold in the liver of MC-treated rats. In addition, monoclonal antibodies to cytochrome P-450 determinants are being used to assess the of P-450 forms. This system provides a new approach for analyzing the biochemical metabolism of the polycyclic hydrocarbon, benzo(a)pyrene.

The first and the obligatory step in the metabolic activation of aromatic amines and amides involves a cytochrome P-450 dependent N-hydroxylation. 2-acetylaminofluorene (AAF) has served as a model compound in numerous studies on the mechanism of aromatic amine carcinogenesis. AAF is extensively metabolized by the cytochrome P-450 isoenzymes both on the nitrogen and on the various carbon atoms. The hydroxylation of the nitrogen, as previously mentioned, is the first step in the metabolic activation of AAF, whereas hydroxylation of the carbons is a true detoxification step since these phenols are neither mutagenic nor carcinogenic. It is now clear that different P-450 isoenzymes catalyze N- and C-hydroxylations, and therefore the relative amount of these P-450 forms may determine the percentage of the AAF dose that is metabolically activated.

Gene cloning and gene transfer techniques have now been applied to investigations on malignant transformation by chemicals. The DNA of 3-methylcholanthrene (3-MC) transformed mouse cells has been shown to possess biological activity when it is transferred into non-transformed recipient cells. The transforming gene in four independently transformed mouse fibroblast lines is apparently associated with a specific sequence of nucleotides. This finding provides the first direct evidence that transforming genes which mediate chemical carcinogenesis exist in chemically transformed cells. DNA from some chemically induced and spontaneous tumors will induce transformation. Although it is not yet possible to conclude that the gene detected is the primary target of the chemical carcinogen, current model systems are based on the idea that genes, which during normal expression are not deleterious and perhaps even vital for normal growth and differentiation, can become oncogenic.

When chemically transformed human fibroblasts and the untransformed parental cells were compared by two-dimensional gel electrophoresis of their proteins, one new polypeptide was recognized in the proteins from a transformed line. This new polypeptide was identified as a product of mutated B-actin gene. Following isolation of the mRNA of this protein and cloning cDNA complementary to the actin mRNA, a recombinant containing human B-actin cDNA was derived. This finding has provided the first molecular evidence for the occurrence of a mutation in chemically transformed cells.

Defective repair of DNA damage is linked to cancer in some cases of genetic predisposition (e.g., xeroderma pigmentosum). Therefore, cellular ability to repair damaged DNA may be important in maintaining cells in a non-malignant state. Recently, human tumor cell strains have been identified as deficient in repairing methylation damage. Strains of normal human fibroblasts were proficient in such repair, including fibroblast strains prepared from people whose tumors gave rise to repair deficient strains. Such repair-deficient cells were killed by low concentrations of alkylating agents including chemotherapeutic agents. Strains both deficient and proficient in the repair of methylation damage were found to have relaxed DNA after methylation treatment, but only the repair-proficient strains could restore their DNA to normal conformation. Other repair-deficient strains were deficient in post-methylation DNA relaxation, and repair-proficient strains showing a slow post-methylation restoration of DNA conformation were somewhat sensitive to methylation killing.

Tumor promoters are a group of chemicals of particular interest since they are capable of enhancing or accelerating the expression of neoplastic potential in latent neoplastic cells. Many tumor promoters differ from classical carcinogens in that they do not covalently interact with macromolecules, are not mutagenic, may be tissue specific, and can induce many transient phenotypic changes in target cells at extremely low exposure levels. Understanding their mode of action is an important goal in carcinogenesis research since the promotion phase of carcinogenesis appears to be reversible. Skin carcinogenesis by classic carcinogens in the mouse is associated with an alteration in differentiation which allows initiated cells to proliferate under conditions where normal cells must terminally differentiate.

In skin cell cultures, phorbol esters produce divergent responses inducing some cells to proliferate and others to differentiate. The net effect of this heterogeneous response is a redistribution of cell types to favor the proliferating population. Cells resistant to terminal differentiation, such as initiated cells, would be expected to increase in number as a result of promoter-induced cell redistribution and to continue to proliferate in tissue regions where normal cells must differentiate. Such clonal expansion of initiated cells could ultimately result in a tumor, initially benign, but with an increased risk for malignant change.

Recent evidence on the mechanism of action of the tumor promoter TPA indicates that it can induce specific changes in target cells rather than general alteration of protein synthesis. The synthesis of five proteins was increased; three of these proteins (m.w. 25,000; 55,000; 70,000) were not synthesized (or synthesized at low rates) in untreated epidermal cells. These findings help define the biochemical changes which occur in epidermal cells during tumor promotion.

Studies have been conducted to identify the sequential progression of genetic or regulatory changes which occur in mammalian cells when they are exposed to exogenous or endogenous tumor promoters. Exposure to 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a tumor promoter, switches off procollagen synthesis pre-translationally and probably transcriptionally. This action is reversible by retinoic acid, known to act post-translationally. Phorbol esters produce specific changes in ganglioside biosynthesis. The binding of phorbol diester to cell membrane binding sites can be blocked by phospholipid methyltransferase and other inhibitors. It has been shown further that phorbol esters stimulate cell growth in culture and have tumor-promoting activity in vivo. Phorbol esters cause a rapid release of fibronectin from cells in culture with concomitant changes in cellular morphology. These studies form a basis for the proposal that TPA and its active analogues probably have some structural resemblance to an endogenous growth-promoting or differentiation modulating substance(s) that has specific membrane receptors.

For a better understanding of the host response to chemical carcinogens, a number of model systems using cell tissue and organ cultures and many different animal species and strains within a single animal species are necessary. Such systems form the basis for tests or bioassays to determine the carcinogenicity of various chemical agents in the environment. While ideally it should be possible to predict risks to man, extrapolation of mutagenicity and carcinogenicity is an extremely complex problem and is being approached cautiously.

Developing new experimental models and more sensitive assays remains an extremely active area of research in carcinogenesis; some examples of in vitro and in vivo models follow.

In Vitro. The serial passage and clonal growth of human bronchial and esophageal epithelial cells in serum-free medium and without feeder-cells provides new opportunities for the study of carcinogenesis and differentiation in these human cells. Such studies would permit related studies on metabolism of chemical carcinogens, DNA damage by carcinogens and transformation of epithelial cells.

A very sensitive immunoassay, ultrasensitive enzymatic radioimmunoassay (USERIA) was developed by combining radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). It is 100- to 1,000-fold more sensitive than RIA in detecting certain virus antigens. The procedure was modified to determine covalent binding of chemical carcinogens to DNA. These assays have already been used to extrapolate carcinogenesis data among animal species. For example, the metabolism of benzo(a)pyrene (BP) by cultured tracheobronchial tissues from different species including human, bovine, hamster, rat, and mouse has been investigated. Although wide quantitative differences in carcinogen binding to DNA were found among the different species and among the different sources of cells within a species, the metabolism of BP is qualitatively similar in tracheobronchial tissues of man and animal species for which this agent is carcinogenic. They also permit detection of DNA-adducts from biological samples exposed to small quantities of BP. Samples of human lung and white blood cells from individuals exposed to BP through lifestyle (smoking) or occupation (coke oven or shale retort workers) are being tested for adducts. The tests offer new possibilities to monitor other clinical material.

A human embryonic lung fibroblast cell strain, MRC-5, can be transformed by a variety of chemical carcinogens. By blocking cells in the G_1 period, a number of chemical agents of a hormonal nature are able to sensitize the cells to transformation when added during early S period. These observations strengthen evidence that DNA metabolism is a central point in the carcinogenesis process and provide a basis for new cellular transformation systems which are pertinent to the study of molecular mechanisms of carcinogenesis.

A positive linear correlation between the induction of sister chromatid exchanges (SCE) and transformation by carcinogens that differ in their mode of interaction with the DNA, shows that SCE and in vitro transformation are related phenomena. SCE induction, a cytological manifestation of DNA damage, may result in random changes of the genetic material responsible for the conversion of a normal cell into a neoplastic cell.

Extracellular calcium concentration regulates epidermal cell proliferation and differentiation in culture. The regulation of differentiation by calcium is not associated with changes in cyclic nucleotide levels but appears dependent on a functioning Na+-K+ ATPase pump. The development of epidermal cell lines with high cloning efficiency has provided an opportunity to study carcinogen-induced change in differentiation using an assay that quantitatively enhances the frequency of this event. The number of foci produced is directly dependent on carcinogen dose, and each focus appears to be clonal in origin.

Using cultures of adult rat hepatocytes on collagen gel-nylon mesh, the precarcinogen 2-acetylaminofluorene and the direct-acting carcinogen methyl methanesulfonate triggered unscheduled DNA synthesis (DNA repair) in the hepatocytes. Moreover, in cells cultured for more than 24 hours, the response to these carcinogens was absent unless dexamethasone, a potent synthetic glucocorticoid hormone, and glucagon were present in the culture medium. These studies not only suggest the potential use of the collagen gel-nylon mesh primary hepatocyte culture as a test for chemical carcinogens, but also demonstrate the importance of specific hormones in maintaining the ability of cultured hepatocytes to repair DNA presumably damaged by these agents.

In Vivo. One such model has been developed for liver carcinogenesis which is based on the hypothesis that an initiating carcinogen induces an altered hepatocyte resistant to the cytotoxic effects of carcinogens. Then, by appropriate selective pressures, the resistant hepatocyte is stimulated to produce nodules. This model is based partly on the finding that a single administration of either diethylnitrosamine or N-methyl-N-nitrosourea to rats whose liver cells were stimulated to proliferate by partial hepatectomy resulted in the induction of altered hepatocytes. These altered cells were resistant to inhibition of their proliferation by dietary 2-acetylaminofluorene, and their appearance was considered to represent the initiation of liver carcinogenesis in these animals. Since they persisted for up to 36 weeks with no decrease in number, the induction of functional resistance was considered to be an irreversible early step in liver cancer.

Since certain chemicals which are ordinarily not hepatocarcinogenic can induce liver cancer in adult rats and mice if given in association with the proliferative stimulus of partial hepatectomy or a single necrogenic dose of carbon tetrachloride, several other liver carcinogens and non-liver carcinogens were tested to see if they also induce functionally resistant hepatocytes. Twenty-one different carcinogens were tested, including 5 polycyclic aromatic hydrocarbons, 5 aromatic amines, 4 N-nitroso compounds, and 7 miscellaneous chemicals such as urethan, safrole, and dieldrin. As controls, 7 different noncarcinogenic analogs were also tested. All of the chemical carcinogens, but none of the controls, induced resistant hepatocytes, suggesting that one of the major factors determining hepatocarcinogenic activity of many compounds may be the presence or induction of cell proliferation at an appropriate time relative to the administration of the compound. These results also suggest that this model system might be developed as a short-term in vivo test for carcinogens.

Although there is substantial epidemiological evidence that benzene causes leukemia in humans, there has been no suitable animal model to study this phenomenon. Recently, it was observed that small numbers of CD-1 mice and Sprague-Dawley rats developed myelogenous leukemia following chronic exposure to benzene vapor. This type of leukemia is often observed in humans exposed to benzene. In addition, a significant number of C57Bl mice which were clinically exposed to benzene vapor developed thymic lymphoma, a rare spontaneous tumor in these animals. These findings indicate that hematopoietic tumors can be produced in certain animals following extensive inhalation exposure to benzene and suggest that such animals may be useful in providing models to study benzene-induced leukemia and lymphoma in humans.

EPIDEMIOLOGY (FIELD STUDIES)

Epidemiologic investigations into the environmental and host determinants of human cancer have been given special emphasis. By compiling statistics on cancer incidence, mortality and survival, and by pursuing multidisciplinary projects that combine epidemiologic and experimental approaches, considerable new information on the etiology of cancer has been generated. To provide a systematic means of identifying risk factors, findings are grouped and reported under categories such as incidence/mortality studies, demographic studies, occupational studies, nutritional studies, family studies, and lifestyle studies.

Incidence and survival information on cancer was obtained from a group of ten population-based cancer registries under the SEER (Surveillance, Epidemiology and End Result) Program. Survival data for patients diagnosed between 1973 through 1979 are now available. Overall, the average annual age-adjusted incidence rate for all forms of malignant neoplasms for all races and both sexes combined was 331.5 per 100,000 population. The most common primary sites of cancer are colon, rectum, breast, and lung with age-adjusted rates of 48.5, 46.7, and 46.7 per 100,000 population, respectively. In fact, these three sites account for 42.7% of all malignancies. The average mortality rate due to malignant neoplasms for all areas is 168.5 per 100,000 population of all cancer deaths; lung cancer accounted for 21.7%, colorectal - 13.6%, and breast cancers - 9.2%. These three sites accounted for over 40% of cancer cases and deaths. Considerable variation is noted in both incidence and mortality from cancer among the various racial and ethnic groups. Striking differences occurred among the race-sex group and geographic areas with respect to incidence of cancer of various sites.

An atlas of cancer mortality was published for the white population, followed by a companion atlas for the non-white population covering the years 1950-69. Maps for non-neolastic diseases have also been prepared emphasizing conditions that predispose to cancer or share etiologic factors. Mapping of the 1970-75 mortality from some of the more common cancers was conducted during this past year. Most striking was the updated map for lung cancer among white males which revealed a shifting geographic pattern compared to the earlier period. Rates were high in broad stretches of the South, and elevated mortality was no longer seen in northern metropolitan centers.

Time trends over the 1950-75 period were examined for several cancers. Analyses by birth cohort showed that the rate of increase in lung cancer was considerably greater among blacks than whites. For males born in the late 1800s mortality in whites exceeded that in blacks by 50%, whereas for those born after 1915 the rates in blacks surpassed those in whites by 50%. Increases in mortality over time were also noted for non-Hodgkin's lymphoma, particularly the histiocytic type, for multiple myeloma, especially among blacks, and for malignant melanoma. Results on cancer patient survival have indicated that white cancer patients had a more favorable survival experience than blacks, part of which was due to a more favorable stage distribution and diagnosis. Largest differences in survival between whites and blacks occurred for cancer of the urinary bladder for each sex and for cancer of the uterine corpus.

Occupational studies have been used as an approach to identifying environmental carcinogens. Agents studied in the workplace may be found in the air, food, water, or consumer products, so that occupational studies maybe helpful in evaluating the risks to the general population. The association between employment in the petroleum industry and cancer mortality, particularly cancer of the brain, has been under intensive investigation. Earlier reports suggested that active workers in the petroleum refining and petrochemical industry experienced high mortality from leukemia and multiple myeloma and cancers of the brain, stomach, and kidney. A recent study of retired workers showed a similar pattern. The relative frequencies for leukemia, multiple myeloma, and non-Hokgkin's lymphoma were significantly elevated and the number of deaths from brain cancer was slightly higher than expected. A proportionate mortality study of other petrochemical plants also revealed an excess of deaths from cancers of the brain and skin.

The study of radiation-induced cancer is also a promising approach to understanding carcinogenesis in general. To characterize the risk of radiogenic breast cancer, the three major sets of human data were analyzed: survivors of the Hiroshima and Nagasaki atomic bombs, tuberculosis patients exposed to multiple chest fluoroscopies, and postpartum mastitis patients treated with x-rays. The findings suggest the following: the risk of breast cancer is greatest in persons exposed as adolescents; the dose-effect relationship is consistent with linearity; fractionation does not appear to diminish risk nor does time since exposure; the interval between exposure and clinical appearance of radiogenic breast cancer is mediated by age-related factors.

Studies of drug-induced cancer have been valuable in the discovery of new carcinogenic agents and in the development of insights into mechanisms of carcinogenesis. The use of high-dose immunosuppressive drugs by kidney transplant recipients was associated with a 25-fold increased risk of lymphoma and lesser excesses of lung cancer, bladder cancer, soft tissue sarcomas, cancers of the liver and bile ducts, and malignant melanoma. Several studies on patients treated with cytotoxic drugs have revealed an excess risk of acute leukemia associated with the use of alkylating agents. The risk appears to follow a dose-response relationship rising to several hundred times that expected among those receiving the highest doses and is unrelated to the primary condition for which treatment was initiated.

Interest in dietary factors associated with the etiology of cancer continues to increase. Heavy alcohol consumption was the dominant risk factor in a case-control survey of esophageal cancer among black men in Washington, D.C., where the mortality rate from this highly fatal cancer exceeds the rates in all other U.S. cities, being higher than the national level for nonwhite males by 2.5- fold and for white males by 7-fold. Nutritional deficiency was also found to play an independent role, with decreased intake of fruits and vegetables, fresh meats, and dairy products. Dietary influences on breast cancer were also suggested by the higher beef and pork consumption found among patients in a case-control study in Canada. The findings may provide support for the notion that high dietary fat is a risk factor for breast cancer.

Analysis of the probability of occurrence of specific cancers in relation to serum cholesterol levels showed that there is a negative relationship between serum cholesterol level and colon cancer. The incidence of colon cancer was high among individuals with serum cholesterol under 180mg/100ml.

Interdisciplinary studies of high-risk families continue to provide new insights into the mechanisms of host susceptibility to cancer. Based on a detailed study of 400 members of 14 melanoma-prone families, the clinical and pathologic features of dysplastic nevus syndrome (DNS) have been precisely defined. Thirty-one new primary melanomas were identified in study participants documenting the value of close surveillance in the detection of early surgically curable lesions. The role of these dysplastic precursors in non-familial melanoma was also established. In vitro ultra-violet (UV) radiation sensitivity in cultured skin fibroblasts of patients with hereditary melanoma and DNS suggests a biologic basis for host-environmental interactions in this syndrome. Genetic analysis indicates that hereditary melanoma segregates as an autosomal dominant trait, a heritable enzymatic defect predisposing to melanoma, manifested as UV sensitivity.

Another study designed to identify the predisposing roles of cancer genes and environmental carcinogens has focused on the genetic disorder known as neurofibromatosis (NF). The data showed that NF is the most common genetic disorder associated with childhood cancer having been found in about 1% of the cases studied. Two forms of cancer, nonlymphocytic leukemia and rhabdomyosarcoma, occurred excessively.

Risk factors associated with breast cancer may be summarized as follows: among women over age 45, late age at first birth, low parity, late age at menopause, and non-breast feeding are linked to increased risk; among women below this age, late age at menarche and recent use of birth control pills were identified as risk factors. Late age at first birth, difficulty in conceiving, previous benign breast disease, and a mother-sister family history of breast cancer are associated with higher risk at all ages.

Although no viruses have been causally implicated in human cancer, epidemiologic information on possible associations continues in two areas. The association of hepatitis B virus with the carrier state and the high incidence of chronic liver disease or primary hepatocellular carcinoma constitute a major health problem of worldwide concern. Although it may take many years to assess its value, a safe virus vaccine exists with the potential for decreasing the morbidity among millions of hepatitis carriers and for determining factors associated with liver cancer. Investigation of the nature of the virus and the molecular characterization of the viral DNA has recently made it possible to demonstrate the integration of viral sequences in liver cell DNA in hepatitis and hepatoma.

The relationship of Epstein-Barr virus (EBV) to Burkitt's lymphoma (BL) is also being investigated. High antibody to EBV has been reported to be a marker of increased risk of developing BL in African children. Males showed a 2-fold excess of BL, but females had higher titers to EBV than males at every age. Whatever factor promotes the excess risk in males appears independent of EBV response. In other studies the EBV titers did not change in BL patients who survived many months (up to 6 months) in remission before relapsing, suggesting this test is not a useful means of predicting relapse.

SUMMARY REPORT

FREDERICK CANCER RESEARCH CENTER

The contractor, Litton Bionetics, Inc., conducts independent research and provides research support to intramural laboratories located at Frederick. For the Division of Cancer Cause and Prevention, the research portion consists of two major efforts: the Biological Carcinogenesis Program (BCP) and the Chemical Carcinogenesis Program (CCP). The work performed in these two areas is broadly directed toward understanding the process of carcinogenesis and interrelates with research programs sponsored by other Divisions of the NCI. The research support portion, Carcinogenesis Intramural Program (CIP), provides services to DCCP intramural laboratories at Frederick.

Biological Carcinogenesis Program: The objectives of the current Program are to identify and describe the complex interactions between oncogenic viruses and host cells, with emphasis on understanding host genetic, hormonal, immune and other environmental factors. Animal systems which provide data and concepts applicable to human cancer are emphasized. The sections within the program investigate the primary structures of viral and transformation-specific proteins; the replication and transcriptional control of transforming genes; and the biology of retroviruses and herpesviruses in relation to the development and control of malignant diseases.

Chemical Carcinogenesis Program: The objectives of the current Program are to investigate the mechanisms of carcinogenesis by various chemicals with particular emphasis on N-nitroso compounds, polycyclic hydrocarbons, aromatic amines and other carcinogens found in the environment. The biochemical activation of chemicals, the comparative metabolism in different animal species and DNA damage and repair -- in general, the molecular aspects of the carcinogenic process, represent major research efforts. Although some groups are concerned with the fundamental aspects of carcinogenesis by chemicals, others have the objective of providing a rational basis for assessing risk of exposure, and involve the improvement and interpretation of biological tests for mutagenesis and carcinogenesis.

Carcinogenesis Intramural Program: This Program was organized to provide DCCP scientists with separate support services: professional, technical and administrative assistance; material and supplies, including viruses and virus products; glassware and media preparation; laboratory animal procurement and maintenance; histologic and electron microscopic assistance; biohazard protection; facility designs and renovations; and other routine custodial services.

Until recently, this Program supported four intramural sections within two laboratories. However, this year the entire Laboratory of Viral Carcinogenesis and the Laboratory of Experimental Pathology were transferred to the Frederick facility. These moves have significantly increased the need for support services. The Division has plans to increase the numbers of intramural scientists at FCRC in the future.

Specific accomplishments of the BCP and CCP are given in the contract report which follows (See: Litton Bionetics, Inc., N01-CO-75380). Reports of DCCP intramural laboratories appear in another section of this Annual Report.

Program Administration and Projections: Contractor-initiated research conducted within the BCP and CCP is reviewed regularly by DCCP and NCI peer groups and program officials. Projects within each program are carefully evaluated to determine relevance, priority and need as well as scientific merit. This year, because of constraints in the NCI operating budget, the level of funding for contractor-initiated research has been reduced by about 30%. These reductions will be effected during FY 1981-1982. Specifically, BCP will (a) phase down production of viral reagents for the extramural program, and (b) phase out its Section on Molecular Biology of Carcinogenesis; similarly, CCP will phase out its In Vitro Carcinogenesis Section. In addition, the contractor will consolidate its efforts to provide additional space for DCCP intramural laboratories that will be transferred to the Frederick facility next year.

LITTON BIONETICS, INC. (N01-CP-75380), BETHESDA, MARYLAND

Title: Operation and Maintenance of the Frederick Cancer Research Center (FCRC), National Cancer Institute, Frederick, Maryland 21701

Contractor's Project Director: Dr. Michael G. Hanna

Project Officer (NCI): Dr. William W. Payne

Assistant Project Officer (DCCP): Dr. Henry J. Hearn

BIOLOGICAL CARCINOGENESIS PROGRAM -- Director: Dr. Ray Gilden

Objectives: The objective of this Program is to conduct research on the complex interactions between oncogenic viruses and host cells leading to the understanding of malignant diseases. The Program has five research sections and a resources section with the following goals:

1. To characterize type C retroviruses using current techniques of molecular biology.
2. To determine the structure of retrovirus transformation-associated gene products.
3. To characterize type B retroviruses, particularly (MMTV).
4. To conduct research on the comparative biology, biochemistry, and oncogenicity of lymphotropic herpesviruses of nonhuman primate origin.
5. To determine the mechanisms of leukemogenesis by murine tumor viruses.
6. To produce large-scale amounts of tumor viruses and/or viral components for use within the FCRC and for distribution to the Biological Carcinogenesis Resources Program.

CHEMICAL CARCINOGENESIS PROGRAM -- Director: Dr. William Lijinsky

Objectives: The objectives of this Program are to investigate the mechanisms of carcinogenesis by various chemicals and to provide a rational basis for risk assessment. The Program has seven sections with the following goals:

1. To elucidate the mechanisms of carcinogenic and mutagenic activity of N-nitroso compounds through studies of their metabolic interactions in biological systems.
2. To determine the relationship between chemical structure and carcinogenicity, particularly nitrosamines and triazenes.
3. To study the capacity for DNA repair and sensitivity to chemical carcinogens in tumor cells from patients with high risk to develop cancer.
4. To describe classes of DNA lesions induced by chemical carcinogens in relation to the ability of the cell to repair damage.
5. To elucidate the mechanism(s) of chemical carcinogenesis using current cytogenetic techniques.
6. To adapt *in vitro* bacterial systems to investigate the correlation between mutagenicity and carcinogenicity of chemicals.
7. To synthesize and analyze chemicals, and develop new methods for the separation, analysis, and characterization of complex organic and inorganic mixtures from biological and environmental samples.
8. To identify chemical compounds potentially hazardous to man.

SUPPORT SERVICES

Pathology and Histopathology; Environmental Control and Research; Animal Resources Program. These services, supported by the DCCP on a cost-sharing basis, are also available to the intramural laboratories through the Carcinogenesis Intramural Program (Dr. Donald Fine, Director).

Objectives: The objective of these programs is to provide support services to all investigators at the Frederick facility. The units above have the following goals:

1. To provide histological preparations, pathologic diagnosis, and to perform animal necropsies.
2. To provide a safe workplace for all laboratory operations.
3. To breed high quality laboratory animals and maintain animal health.
4. To provide professional, technical and administrative support for the following intramural laboratories now located at Frederick: Laboratory of Molecular Virology - one section; Laboratory of Viral Carcinogenesis - seven sections, two units; Laboratory of Cellular and Molecular Biology - two sections; Laboratory of Experimental Pathology - 4 sections.

Major Findings: Contractor-initiated research funded by DCCP:

Molecular Biology of Retroviruses. Rat viral srcs and endogenous, cellular srcs constitute a family of genes which can be distinguished by nucleic acid analysis.

The Rasheed strain of rat sarcoma virus codes for a fusion product which covalently links endogenous rat gag gene and the p21 gene of Harvey and Kirsten sarcoma viruses.

The reticuloendotheliosis virus (REV) contains an avian-derived, cellular insert, the putative transforming gene, and helper virus structural components. Studies are underway to identify the transforming gene product of REV.

Recently isolated rat sarcoma virus and REV src hybridization probes will be used to determine whether activation of endogenous sequences is involved in the spontaneous cellular transformation.

Cloned baboon viral genomes show considerable heterogeneity in restriction mapping and infectivity. Subgenomic fragments will be used to search for related sequences in human DNA.

A defective murine leukemia virus which produces only env products in infected cells will be useful for studying env synthesis independent of gag and pol products.

Immunochemistry. Peptide analysis of the gag polyprotein precursor, pr65 of Rauscher leukemia virus, has been completed thus providing a reference standard for many laboratories.

Peptide analysis and immunoassays have established that the p30 of REV is closely related to a similar protein of endogenous viruses of macaques.

Peptides corresponding to putative cleavage sites in viral polyprotein precursors have been synthesized and used as substrates to determine the precise nature of cleavage sites and as absorbants for purification of cleavage enzymes.

Molecular Biology of Carcinogenesis. Monoclonal antibodies to gp52 determinants clearly distinguish several mouse mammary tumor virus (MMTV) isolates. Radiolabled antibodies to this component attach *in vivo* to the surface of mammary tumor cells. Antibodies from several clones revealed distinct bonding sites. Some antibodies inhibited or neutralized the focus-forming capacity of a KiSV (C_3H-MMTV) pseudotype; others blocked the reaction. Thus, functionally distinct antibodies could account for viral persistence in the presence of neutralizing antibodies.

Oligonucleotide sequences at the 5' terminal of several mammalian type C retroviruses are highly conserved. Analysis of this region provides an additional method for identifying intergroup relatedness among these viruses.

Primate Virus Immunobiology. Using fluoroimmunoelectrophoretic analysis, EBV nuclear antigen (EBNA) is a protein between 65_K and 73_K. Another antigen, an 81_K protein, may also be associated with this antigen.

Monoclonal antibodies, which neutralize EBV, react with a membrane antigen (MA), an 86_K protein.

A linkage map of *H. papio* DNA was constructed; the genome structure was similar to that of EBV and colinear with it.

An enzyme-linked immunoabsorbent assay (ELISA) was developed to measure EBV antigens.

Monoclonal antibodies to several antigens present on human lymphocytes, cross-react completely with gorilla and chimpanzee cells. Surface antigens of other nonhuman primates possess considerable diversity.

T-cell growth factor from a gibbon cell line has the same biochemical properties as the human factor and supports the T-cell growth of human, ape, monkey, rabbit, and mouse origins.

Molecular Mechamisms of T-Cell Leukemogenesis. A new lymphokine (IL-3) was identified and purified. This factor is important in the establishment of T-cell cultures and greatly amplifies the blastogenic responses of preleukemic mice.

Viral Resources. The Viral Resources Laboratory produced, purified and characterized 450 liters of retroviruses per week: DCCP/BCB - 240 liters/wk; FCRC/BCP - 180 liters/wk; and FCRC/CIP - 30 liters/wk. In contract year 1981 (October 1980 to present), commitments included: DCCP/BCB - 180 liters/wk; FCRC/BCP - 180 liters/wk; and FCRC/CIP(LVC) - 90 liters/wk. Throughout this period, all large-scale production commitments were fulfilled in a timely manner with purified materials derived from a large number and variety of producer cell lines; 21,310L of retrovirus containing cell culture fluids were produced; 20,539 ml of virus concentrate were distributed. Additionally, the developmental biology group within the Viral Resources Laboratory continued to support the production and purification groups and other FCRC/BCP sections in characterizing and storing cells. The group performed cell culture oriented research projects, including the development of monoclonal antibodies to a wide variety of antigens in collaboration with multiple FCRC programs as well as outside investigations.

Mechanisms of Nitrosamine Carcinogenesis. Morpholine fed to rats together with nitrosopropyline, nitrosohydroxyproline, nitrosophenylbenzylamine and nitroso-N-methylpiperazine has resulted in the deaths of animals with liver tumors induced by the nitrosomorpholine formed in the alimentary tract. Concurrent administration of sodium thiocyanate, which is a catalyst for transnitrosation in chemical systems, increased the incidence of liver tumors. Nitrosophenylbenzylamine gave rise to a high incidence of esophageal tumors in rats on chronic feeding.

The qualitative correlation between carcinogenicity and mutagenicity of N-nitroso compounds was good; however, more than 20 carcinogenic nitrosamines were nonmutagenic when activated with rat liver microsomes. Six of these compounds induced tumors in the liver of rats when administered chronically.

The *trans* isomer of nitroso-2,6-dimethylmorpholine is more a potent esophageal carcinogen in the rat than is the *cis* isomer. However, in the Syrian hamster and the guinea pig, the *cis* isomer is more potent than the *trans*. The results suggest that the mechanisms of cancer induction in these species are different.

In comparing the carcinogenic effectiveness of a series of nitrosomethylalkylamines in rats, the even-numbered carbon chains were induced bladder tumors, while the odd-numbered chains gave rise to liver or esophageal tumors, or both.

Ultrastructural Studies in Chemical Carcinogenesis. Mucous cells in the upper airways and Clara cells in the lower airways of Syrian hamsters have been identified as the principle sites of binding and metabolism of nitrosodiethylamine (DEN). In the fetal hamster trachea, mucous and nonciliated stem cells were the binding sites. The level of cellular differentiation is the major factor determining the amount of binding in this system.

Nitrosoheptamethyleneimine (NHMI)-induced squamous cell carcinomas in the rat lung originate from basal cells, and a new early marker for carcinogenesis was identified after four weeks of carcinogen treatment.

The mitochondria of periportal hepatocytes were shown to be the principle site-of-binding of [^{3}H]-methapyrilene in the rat liver. Proliferation of mitochondria in these cells was revealed as an early specific marker of methapyrilene-induced liver tumors.

In the hamster pancreas the zymogen granules, rough endoplasmic reticulum, and heterochromatin of acinar cells were identified as the principle intracellular sites of binding of N-nitroso-2,6-dimethyl-morpholine (DMNM). Squamous cell carcinomas induced in the rat urinary bladder by N-nitroso-N-methyl-N-dodecylamine (NMDA) arise from pre-existing transitional cell tumors.

Chemistry of Carcinogenesis. Although the metabolism of dimethylnitrosamine (DMN) and N-nitroso-N-methylaniline (NMA) can proceed by the α-hydroxylation pathway, denitrosation and reduction occur simultaneously and predominate α-hydroxylation proceeds more efficiently *in vivo* than *in vitro* systems.

Formaldehyde formation is not an accurate indicator of nitrosamine demethylation because S9 liver preparations oxidize formaldehyde to formate.

Female Fischer rats given methyl-2-hydroxy-ethylnitrosamine develop hepatocellular carcinomas within 50 weeks. A homolog, methyl-3-hydroxypropylnitrosamine, failed to produce any tumors after two years.

Decomposition of trialkyltriazenes follows acid catalysis kinetics leading to the formation of alkyldiazonium ions; these ions are putative ultimate carcinogens produced during the metabolism of dialkylnitrosamines.

Molecular Aspects of Chemical Carcinogenesis. 7,12-dimethylbenz(a)anthracene-DNA adducts are (a) more difficult to release by enzymatic digestion *in vitro*, (b) more slowly excised in cell culture systems, and (c) modified at guanine residues rather than adenine residue -- factors that may be related to the high carcinogenicity of this compound.

Studies of interactions between hydrocarbons and DNA have confirmed that the nature of products formed is dependent on substrate concentration when using rat, hamster, or mouse liver microsomal preparations. However, if intact cells are used, the nature of the products is independent of hydrocarbon concentration. Only intact cellular systems reproduce the binding which occurs during initiation of carcinogenesis in target tissues.

DNA Repair Studies. A DNA repair phenotype Mer-, characteristic of about 20% of the human cell strains was identified. This strain is deficient in supporting the growth of adenovirus-5 preparations treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), but supports the growth of nontreated viruses. In comparison to Mer+ strains, Mer- strains exhibit greater sensitivity to reproductive killing as assayed by colony forming ability and perform more DNA repair synthesis after MNNG-treatment. Mer- tumor cell strains are hypersensitive to other chemical alkylating agents: methyl methanesulfonate and bis-1,3-(2-chloroethyl) 1-nitrosourea; however, their relative sensitivity to MNNG is greater. Using high pressure liquid chromatography techniques, Mer- strains are deficient in removing O^6-methylguanine from DNA after MNNG treatment, while Mer+ cell strains are not. Mer- tumor cell strains gave rise to detectable levels of sister chromatid exchanges at much lower concentrations of MNNG than Mer+ strains.

SV40-transformed human fibroblast cell strains were tested to determine whether the Mer- phenotype was characteristic only of certain strains derived from human tumors. Of eleven SV40-transformed strains, seven were found to be Mer- and 4 Mer+. All SV40-transformed Mer- strains tested failed to excise O^6-methylguanidine as efficiently as the SV40-transformed Mer+ strains.

Cell strains derived from patients with hereditary primary neuronal disorders showed hypersensitivity to MNNG-induced reproductive killing in fibroblasts derived from Huntington's disease (6 strains), familial dysautonomia (4 strains), tuberous sclerosis (3 strains), infantile spinal muscular atrophy or Werdnig-Hoffman disease (1 strain), and spinocerebellar ataxia (1 strain). These studies were conducted using coded and non-coded cell strains. For comparison, 13 fibroblast strains derived from normal control donors were also studied. The 15 strains derived from the neurological disease patients showed hypersensitivity to MNNG killing. Whether or not hypersensitivity to DNA-damaging agents is related to the pathogenesis of these diseases, enhanced sensitivity to MNNG may prove useful for the presymptomatic detection of primary neuronal degenerations.

Seven triazenes were tested for relative toxicities in normal human fibroblast strains. The relative toxicities and mutagenic potencies of the different compounds are generally consistent with chemical reactivity, but not with mutagenicity (Ames test).

In Vivo Carcinogenesis. Each standard protocol bioassay was performed in four main phases: (1) acute toxicity testing, (2) preliminary dose-finding studies, (3) carcinogenicity testing and (4) data eveluation. These phases are sequential, preprogrammed, completed as a series and may take over four years to complete.

During FY 1980, seven chemicals (Telone, Mirex, cyclohexanone, benzyl chloride, styrene oxide, nitrosodiethanolamine, and methapyrilene) were tested. Telone and Mirex bioassays were terminated on schedule early in 1980. Tissues from animals on these bioassays have been processed and are currently being evaluated. The cyclohexanone bioassay is being terminated on schedule and the animals killed. Benzyl chloride and styrene oxide are in the second year of carcinogenicity testing. The preliminary toxicity phase of two bioassays unequivocally demonstrated that nitrosodiethanolamine and methyapryrilene are carcinogenic for the liver of rats; further testing is unnecessary.

Funding of the following projects, programs was shared by DCCP:

Pathology and Histotechnology. A total of 3,515 animal necropsies were performed (1,080 necropsies for the Bioassay Program and 2,435 necropsies for the Nitrosamine Carcinogenesis Program). The tissues from these animals generated 41,574 stained microslides (16,738 slides for the Bioassay Program and 24,836 slides for the Nitrosamine Carcinogenesis Program). These slides were diagnosed by the staff pathologists. During this time, a total of 1,500 individual animal pathology reports for the bioassay studies and 1,720 individual pathology reports were prepared.

Environmental Control and Research Laboratory. The Environmental Control and Research Laboratory provides safety policies, training programs, evaluations and medical services for workers in all laboratories located at the Frederick facility. Additionally, research is performed to assess the risk of common laboratory procedures, in particular for techniques required for degrading and disposing of chemical carcinogens. This year the group cooperated with members of the International Association for Research on Cancer in devising a procedure for destroying nitrosamines safely and with members of the American Society for Testing and Materials in developing tests to measure the penetration of solvents in protective clothing. The Safety Information Group organized a symposium on "Management of Hazardous Chemical Wastes in Research Institutions."

Animal Resources Program. A completely automated system was developed and implemented to coordinate information relating to animal production and distribution. The production of gnotobiotic animals was expanded. Twelve strains of rederived rodents are now maintained in barrier production colonies. The production of mice, in particular nude mice, has doubled during the current period while rat production remained constant. All mice and rats kept were free of detectable Salmonella, mycoplasma, common internal and external parasites, and pathogenic murine viruses. The diagnostic section continued its surveillance of incoming animals, meeting the objective of maintaining high quality animals for cancer research.

Significance to Biomedical Research and the Program of the Institute: Investigator-initiated research conducted by Litton Bionetics at the FCRC is directed to two major themes -- biological (viral) carcinogenesis and chemical carcinogenesis. The wide variety of scientific activities under these programs provide new information and concepts that are applicable to understanding and controlling cancer in man.

For example, studies on animal tumor viruses provide a means for identifying the mechanisms of malignant transformation at the molecular level because (a) tumor viruses contain and transport genetic information which may initiate derangement of metabolic pathways of normal cells; and (b) tumor viruses may activate cellular genes which are involved in the control of cell growth and cell maturation. Studies on human tumor viruses provide direct information on the action of viruses on specific populations of cells, particularly lymphocytes.

Similarly, studies on chemical agents provide descriptions of the metabolism of chemicals to biologically active forms, chemical structure in relation to mutagenic and carcinogenic activity, and interaction of carcinogens with cellular DNA. Such information will permit the development of reliable transformation assays and strengthen predictions on risks to developing cancer in humans exposed to certain chemicals in the environment.

Proposed Course: Contractor-initiated research conducted within the BCP and CCP is reviewed regularly by DCCP and NCI peer groups and program officials. Projects within each program are carefully evaluated to determine relevance, priority and need, as well as scientific merit. This year, because of constraints in the NCI operating budget, the level of funding for contractor-initiated research has been reduced by about 30%. These reductions will be effected during FY 1981-1982. Specifically, BCP will (a) phase down production of viral reagents for the extramural program and (b) phase out its Section on Molecular Biology of Carcinogenesis; similarly, CCP will phase out its In Vitro Carcinogenesis Section. In addition, the contractor will consolidate its efforts to provide additional space for DCCP intramural laboratories that will be transferred to the Frederick facility next year. As planned, this contract will be readvertised and renegotiated next year. See report of OD, NCI for more specific information.

Date Contract Initiated: September 26, 1977

Current Annual Level: $15.9 million (includes award fee, shared costs, and
Interagency Agreement)

Microbiological Associates (N01-CP-33248), Bethesda, Maryland

Title: A Holding Facility for Small Laboratory Animals

Contractor's Project Director: Dr. Marshall Dinowitz

Project Officer (NCI): Dr. Henry J. Hearn

Objectives:

A. Maintain breeding colonies of BALB/c and C57 BL/6 mice.

B. Maintain a colony of BALB/c mice for studies on the effect of aging on various immunological and virological parameters.

C. Maintain athymic (nude) mice for NCI investigators; monitor the animals.

D. Inoculate mice with hybridoma cells for the production of large amounts of fluids and cells. Provide long-term storage for cell preparations.

E. Inoculate mice and rats with herpes simplex virus and monitor these animals.

F. Maintain and inoculate rats with tumor virus preparations as directed.

Major Findings:

A. Breeding Colonies of BALB/c and C57 BL/6 mice

1. BALB/c. A breeding colony of BALB/c mice sufficient to produce 200-400 newborns per months was maintained. This production colony provided the necessary animals for experiments described below for NCI investigators and for continuous addition to the aging mouse colony which was also used in subsequent experiments.

2. C57 BL/6. A breeding colony of C57 BL/6 mice sufficient to produce 100-300 newborns per month was maintained for use in NCI experiments and for continuous addition to the colony of aging mice.

B. Colonies of Aging BALB/c and C57 BL/6 mice

1. BALB/c. This colony consisted of both male and female mice which ranged in age up to 30-35 months old. These mice were provided in the age ranges required for specific experiments used in assessing the age-dependent virological and immunological characteristics of this mouse strain.

(a) Spontaneous tumors (primarily lymphoid), normal spleens, thymuses, bone marrow, circulating lymphocytes, and tissues from mice with spontaneously developing tumors, e.g., spleen, thymus, bone marrows, circulating lymphocytes, serum were provided to NCI scientists.

(b) Mice in selected age ranges were immunized with virus and viral antigens for the assessment of immune responses (cellular and humoral).

2. C57BL/6. This colony consisted of both male and female mice which ranged in age up to 31 months, used for the assessment of immune responses. Tissues from both normal and tumor-bearing mice were provided to NCI investigators.

C. Athymic (Nude) mice. A facility was maintained for the housing of nude mice under conditions designed to reduce the risk of introducing infectious diseases. Air entering the facility was filtered through HEPA filters and a work hood with HEPA-filtered air continuously "scrubbed" the room air.

Approximately 200 nude mice were received bi-weekly (approximately 5,200 per year) and were inoculated with tumor cell lines for an NCI investigator. Records were maintained on tumor development for approximately 8-9 weeks for each group of mice.

D. Hybridoma fluid production. Hybridoma cell clones were propagated in conventional and nude BALB/c mice as requested by NCI. Ascites fluids were collected weekly, or more frequently as required, processed to remove cells and fluids provided to NCI investigators. Records of total ascites fluid provided per clone and animal utilization records were also provided. In addition, back-up samples of hybridoma clones were frozen and stored in liquid nitrogen.

E. Herpes simplex virus (HSV) experiments.

1. Antiserum production. Thirty to fifty BALB/c mice were immunized with HSV antigens weekly as requested by NCI. Weekly orbital bleeds were performed and sera were prepared, pooled and provided to investigators as requested.

2. HSV latency studies. Approximately 135 neonatal litters of rats and mice were provided for inoculation by NCI investigators with HSV preparation. The virus-challenged neonates were maintained in germ-free isolators until weaned in order to reduce the risk of HSV infection of personnel and other experimental animals. Selected mice and rats were provided to the investigator as requested. The remaining animals were maintained for long-term observation.

F. Production of antisera and immune cells following challenge of rats with RNA tumor viruses, viral antigens, tumor cells and subcellular preparation. A census of 150-400 rats was maintained in a series of experiments designed to elicit specific immune responses to the aforementioned materials. Rats were challenged with virus, viral antigens, tumor cells and tumor cell extracts and palpated for tumor development, bled, and sera were prepared. Tumors were removed and frozen and/or passaged *in vivo*; specific organs were removed and provided to NCI investigators as directed.

G. Antiserum against HSV and HSV antigens. Six to twelve rabbits were challenged with antigen and bled on a periodic basis to provide antiserum to NCI investigators.

H. Antiserum against RNA tumor viruses and tumor virus-induced tumors. Adult rabbits and several litters of neonatal rabbits were challenged, monitored for tumor development and bled for production of antiserum, which was provided to NCI investigators.

Inasmuch as government facilities are available at the Frederick Cancer Research Center for NCI investigators involved in this work, it is expected that this contract will be fully terminated on or before September 1981.

Date Contract Initiated: February 1, 1973

Current Annual Level: $154,899

REPORTS ON INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1981)

The Division of Cancer Cause and Prevention participates in several of the major international agreements on cooperation in cancer research: US-USSR (1972); US-France or NCI-INSERM (1972); US-Japan (1974); US-Italy (1979); US-Germany (1979); and US-Peoples Republic of China (1980).

Collaborative efforts include studies in cancer epidemiology and chemical, physical and viral carcinogenesis, with emphasis on factors related to the etiology and prevention of cancer. Basic and applied research also is conducted in foreign institutions under grants, contracts and cooperative agreements administered through the Divisons's extramural programs.

US-USSR Agreement. Studies conducted under this long-standing agreement are implemented by the Cancer Virology Working Group. Exchange visits of scientists from both contries still provide opportunities for collaboration. Although no joint meetings were held this year to discuss progress in tumor virus research, a symposium on tumor viruses has been tentatively planned for 1981.

In general, the accessibility to a large colony of baboons remains the major contribution to the U.S. These animals have been a valuable source for: (1) isolating primate retroviruses; (2) isolating primate herpesviruses and determining their role in the development of certain lymphoproliferative diseases; and (3) identifying genetic factors resulting from inbreeding. The results of many of these studies have been published in U.S. and Soviet journals and in four proceedings of US/USSR symposia on "Viruses in Cancer and Leukemia".

The Director, NCI has planned to review all cancer activities under this Agreement in September, 1981. Because of increased emphasis on understanding cancer causation, this area may be expanded to include additional basic research in carcinogenesis; such projects would continue to favor a policy of scientist-to-scientist exchanges.

US-France or NIH/INSERM Agreement. In 1979, NIH and INSERM officials met to review the progress of this cooperation, which had existed for several years. At the meeting, the Director, NCI and the Director General, INSERM reduced the number of working groups from three to two; clinical research and basic research. The Basic Research Working Group met with its French counterpart to discuss the conditions for the selection of projects and investigators and agreed that any non-clinical study of scientific merit relating to carcinogenesis could be considered for support. The following areas of interest were noted: cell growth factors, cell differentiation, molecular genetics, tumor promotion, DNA-repair mechanisms, transformation by DNA and RNA tumor viruses, and chemical agents.

Last November, INSERM invited the U.S. Working Group to participate in the first joint meeting under the new organization. The ground rules and procedural guidelines for exchange visits were defined: (1) any non-clinical research relating to the understanding of the process of carcinogenesis would be relevant to the program; (2) only projects of the highest scientific merit would be considered; and (3) participants would be required to (a) justify the need for the actual exchange of scientists from one laboratory to another; (b) explain clearly the contribution of each laboratory to the cooperation; and (c) describe what work would be accomplished within a specific time period. Thus far, several U.S. and French investigators have been approved as exchange scientists. Next year, a bibliography of coauthored publications resulting from this Agreement will be collected and published.

U.S.-Japan Agreement. This long-standing Agreement was also restructured in 1979 to accomodate changes in emphasis of cancer research. The present organization consists of four broad program areas; Eitology, Cancer Biology and Diagnosis, Cancer Treatment, and Interdisiplinary Research. The major objective of etiology research is to provide a fundamental basis for understanding the causation of human cancer, including: epidemiology, viral carcinogenesis, chemical carcinogenesis, and genetics. This year, several members of this Division participated in workshops on "Biochemical Epidemiology" and "Interspecies Correlations in Chemical Carcinogenesis". During the latter meeting, such topics as carcinogen metabolism, mutagenesis and carcinogenesis in different animal species, and the multistage precess of carcinogenesis were presented and discussed. Under the auspices of the Interdisciplinary Group, our scientists organized a meeting on "Differences in Lymphocytic Diseases in the U.S. and Japan". The participants planned several collaborative studies on ethnic differences in lymphocyte-related diseases. Scientists from DCCP also participated in a joint meeting on "*Src* and *Leuk* genes" sponsored by the Cell Biology Program Area.

This Agreement remains one of the most active, and many exchange visits were arranged for U.S. and Japanese scientists under the auspices of this subarea. This binational program is especially suited to the study of malignancies that differ markedly in their occurrence within the two countries.

U.S.-Italy Agreement. Research pertinent to this Division is included in the Cancer Prevention Program. Although the program includes most areas of basic research relating to carcinogenesis, the early activities of this program have been concerned primarily with projects in cancer epidemiology. A workshop which will focus on cancer risks in the workplace is planned for next year.

U.S.-Federal Republic of Germany. This agreement primarily concerns cooperation in environmental carcinogenesis. The U.S. Working Group has agreed to conduct joint investigations on the mechanisms of carcinogenesis, including prevention and modulation of the process.

U.S.-Peoples Republic of China. This newest initiative differs from other binational health programs in that it has no joint steering committee. Cancer epidemiology has been given the highest priority for collaborative research. Molecular biology and related disciplines will await the training of the younger Chinese scientists. Of particular interest to the DCCP are joint studies on the epidemiology of esophageal, nasopharyngeal and hepatocellular carcinomas. Members of this Division have already begun collaborative projects to establish culture conditions for human explants of these organs.

Composition of the Working Groups. The chairmen and members of the U.S. and foreign working groups and steering committees have not changed appreciably this year. More detailed information on the composition of these groups may be found in last year's Annual Report and in the current report of the Office of International Affairs, OD, NCI.

SUMMARY REPORT

SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

October 1, 1980 through September 30, 1981

A. Mission

This program unit comes under the Office of the Director of the Division of Cancer Cause and Prevention where it serves as a focal point for development of program interests and activities and collaboration (intramural and extramural) in the area of environmental cancer. Overall this involves working with federal and state agencies, industrial and academic institutions, trade unions, consumer groups and scientific societies. This office serves as the primary information resource for the Division and the Institute on the role of environmental pollution in carcinogenesis. These coordinated efforts fulfill one of the essential functions prescribed in the National Cancer Act of 1971 as amended by the Biomedical Research and Training Amendments of 1978.

This office has introduced and emphasized the concept of a holistic approach to assessment of stress from environmental contaminants identified as carcinogens in environmental media (air, water, diet, drugs, cosmetics and the workplace) as contrasted with traditional assessment of singular exposures. This involves collaboration with EPA, NIOSH, FDA, OSHA and CPSC relevant to man's multiple exposures. These activities are extended through support of workshops, symposia, presentations at scientific meetings, and publication of reports and proceedings on environmental carcinogenesis.

The activities of the Interagency Collaborative Group on Environmental Carcinogenesis (ICGEC) sponsored by this office, now in its eighth year (58th meeting in June, 1981), reflect one of the mission items of the above referenced legislation. This interagency group encompasses 28 agencies and subagencies. This group, serving as an NCI mechanism for dissemination of information and data on environmental cancer, also functions as a catalyst for program initiatives in stimulation of cooperative work among these agencies and NCI.

This unit also monitors and manages the NCI/EPA and NCI/NIOSH collaborative, contractually supported, programs on environmental and occupational carcinogenesis mandated by OMB and the U.S. Congress in 1977. This program group is a primary interface working with the National Toxicology Program in NCI chemical selection for testing for carcinogenesis and reporting to industry and trade associations on results of testing of these chemicals for carcinogenicity.

B. Other Assigned Functions

This office continues to support the Chemical Selection Working Group (CSWG) of NCI and the Chemical Evaluation Committee of the National Toxicology Program (NTP), the support provided contractually and by staff. The office also maintains a Chemical Selection Planning Group, working with a contractor, which plans agendas and makes prior decisions on chemicals to be submitted to the CSWG. This office is involved in a number of collaborative information research contracts with such organizations as SRI International, Menlo Park, California, The International Agency for Research on Cancer (IARC; Lyon, France), Technical Resources, Inc. (Rockville, Md.), the Department of Energy through the Mitre Corporation, Brookhaven National Laboratory, CDC (Center for Disease Control) and the large collaborative program on environmental carcinogenesis with the Environmental Protection Agency and an occupational carcinogenesis program with the National Institute for Occupational Safety and Health. Through the BOA (Basic Ordering Agreement) mechanism, information resources, in the form of documentation on carcinogens in drugs and cosmetics, are being developed.

C. Accomplishments

Interagency Collaboration

One of the visible mechanisms for collaboration is through the Interagency Collaborative Group on Environmental Carcinogenesis (ICGEC), which meets at intervals of 6-8 weeks. Topics of meetings held this year are as follows: 1) Diet, Nutrition and Cancer Program Working Group; 2) Wood Carcinogenesis; 3) The Movement of Toxic Substances in the Environment; 4) Chemical Waste Incinerator Ships; and 5) Health Hazards of Vapors Encountered in Marine Transportation. In addition, it is expected that one or two more meetings of this group will be held during this reporting period.

The ICGEC-supported task group on the development of a National Data Base on Multimedia Exposure and Body Burden of Environmental Chemicals in Humans has become a continuous effort with the support of an NCI/EPA contract which produces documentation for dissemination to NCI and EPA staff and other agencies, as well as many academic institutions and state health departments.

The contract project with IARC has provided for the usual working group meetings in Lyon, France. Volumes 24, 25, 26 and 27 will issue during this reporting period. As to the IARC Information Bulletin on the Survey of Chemicals Being Tested for Carcinogenic Activity, Volume 9 is being published and will be distributed.

This office has continued participation in the work of the Task Force on Environmental Cancer and Heart and Lung Disease. Chairmanship of one Working Group on Exposure and Metabolism was provided by Dr. Kelsey of this office. During the early part of the year, this working group, consisting of federal and academic scientists, prepared a superior document of 72 recommendations with the rationale for proposed research projects and strategies to be considered which was submitted at a plenary session of the Task Force for study and approval by DHHS and EPA. In addition to an Executive Summary, the scientific document will be forwarded to the U. S. Congress. Report of progress of this Task Force is required annually by Congress.

Workshops, Collaborative Meetings, and Scientific Meetings

This office receives a considerable volume of requests for presentation of papers at scientific meetings and preparation of chapters for books, as well as lectures on environmental cancer. We have participated in organizing committees for meetings, workshops and seminars. During the year, the staff members participated in meetings as presenters of papers or session chairman at the following: Interagency Meeting on Risk Assessment at Oak Ridge, Tennessee, October, 1980; Symposium on Safety Assessment of Artificial Sweeteners, Washington, D. C., May 1981; VI International Bile Acid Meeting: Bile Acids and Lipids; Falk Symposium No. 29, Freiburg, West Germany; and the 1981 International Conference on Residential Solid Fuels, Portland, Oregon.

Lectures were given at the George Washington University Medical School, November, 1980; Mid-Atlantic Association of Cosmetics Chemists, April, 1981; Franklin and Marshall College, Lancaster, Pennsylvania, April, 1981; Wheaton Maryland Library Group, May, 1981; and the Society of the Plastics Industry, May, 1981.

The primary workshops were the Second Annual NCI/EPA/NIOSH Collaborative Workshop: Progress on Joint Environmental and Occupational Cancer Studies, September, 1981 (Proceedings to be published) and the Workshop on Exposure and Metabolism of the Task Force on Environmental Cancer and Heart and Lung Disease, January 1981.

Management of Interagency Programs

During the year, we were able to add some new projects to the NCI/EPA and NCI/NIOSH Collaborative Programs. These programs require considerable interfacing and some measure of public relations. Our first Proceedings of the NCI/EPA/NIOSH Workshop, an 863 page document, was issued and distributed widely. We feel that these proceedings and our published annual reports are benchmarks of managerial achievement.

Interfacing with Trade Associations, Industries and Trade Unions

This office has continued to be responsive to requests from industry and the trade associations for guidance and assistance in animal model selection, protocol design and development, and exchange of data and information. There are frequent interactions with approximately 40 trade associations and 200 individual industrial companies, most of which are initiated by the associations and companies as one mechanism to obtain government guidance for their projected expensive toxicological efforts. There has been an increasing number of queries from law firms and a variety of municipal, state, and federal agencies tracking the government's toxicological activities. Product liability suits, pending legislation, concerns for environmental impact, etc., have magnified the interest in the results of government-sponsored carcinogenicity tests and require accurate, thoughtful responses based on sound scientific evaluation. It is especially necessary to qualify apparent results when dealing with lay individuals.

With the aim of eliminating costly duplication and the use of limited, valuable resources, we have attempted to establish international cooperation in the area of chemical testing. Focal points have been identified in the German, Dutch, English and Norwegian industrial communities, and industrial communities in Sweden, Denmark, and Canada. In some cases, the concerned elements of government in certain of these nations are contacted--directly or through their industrial organizations. Representatives of these communities are routinely supplied with the list of chemicals on, or projected for, test in the carcinogenesis testing program, as well as the prepublication technical reports. In many cases there are face to face or telephone conversations necessary to clearly communicate explanations of specific details in an adequate manner to foreign nationals unfamiliar with our terminology and scientific protocols.

Special Projects on Food Contaminants, Air Pollutants, Water Contaminants, and Drugs and Medical Devices in Environmental Carcinogenesis

Information resource projects are designed to provide timely and authoritative reports on carcinogens in man's environment. This year a report entitled "Biorefractories in Water - Carcinogens, Mutagens and Promoters" was prepared and distributed. As a result of collaboration with NCI, EPA and SRI, a similar report was made available on carcinogenic air pollutants (soon to be published) and a paper was given at the International Conference on Residual Solid Fuels (Portland, Oregon, Kelsey et al). As previously mentioned, under a BOA, two projects are underway to prepare documents for ultimate distribution relevant to monographs on each chemical identified as a carcinogen and/or mutagen in drugs and cosmetics. An update of our report issued last year on water contaminants worldwide, relating to bioassay, epidemiology, identification and classification, will appear this year. An additional feature is a comprehensive tabulation of exposure levels of these carcinogens in water.

Another cooperative project between the National Cancer Institute and the National Toxicology Program is the preparation of a list of chemicals for testing and research for carcinogenesis. This requirement is imposed by the U. S. Congress under the Biomedical Research and Training Amendments of 1978. This task was completed this year. In response to paragraph 4D of the amendments Sections I and II, another cooperative project, providing information regarding the extent to which each Federal exposure standard on a substance decreases the risk to public health from exposure to that substance and how the Secretary or others responded to requests, was completed. These listings, submitted annually, comprise several hundred chemicals.

Committees and Subcommittees

Staff of this office continue to serve on various committees and subcommittees, some within NCI and others interagency. During this year the active work has been confined to the following: DOE Planning Committee for Symposium on Health Risk Analysis; Interagency Collaborative Group on Environmental Carcinogenesis; EPA/DHHS Task Force on Environmental Cancer and Heart and Lung Disease and Subcommittee on Exposure and Metabolism; Chemical Selection Working Group (NCI); Chemical Evaluation Committee (NTP); DOD Committee on Food Program; Chemical Industry Institute of Toxicology Scientific Advisory Panel; Animal Care Committee, NTP; Biological Cancer Resource Contracts Review Group; Interagency Committee on Stratospheric Ozone Protection; and the National Academy of Sciences Committee on Nitrite and Alternative Curing Agents in Foods.

Publications

Kelsey, M. I.: Studies of neutral steroid and bile acid metabolites having activity in short-term tests for carcinogenicity. Proceedings of the VI International Bile Acid Meeting: Bile Acids and Lipids; Falk Symposium No. 29, Freiburg, West Germany, October, 1980.

Kelsey, M. I., Molina, J. E., Huang, S. -K., Hwang, K. -K.: The identification of microbial metabolites of sulfolithocholic acid. Journal of Lipid Research. 21: 751-760, 1980.

Ward, J. M., Reznik, G., Stinson, S. F., Lattuada, C. P., Longfellow, D. G., and Cameron, T. P.: Histogenesis and morphology of naturally occurring prostatic carcinoma in the ACI/segHapBR rat. Laboratory Investigation, 43: 517-522, 1980.

Helmes, C. T., Sigman, C. C., Malko, S, Atkinson, D. L., Jaffer, J., Sullivan, P. A., Thompson K. L., Knowlton, E. M., Kraybill, H. F., Hushon, J. and Barr, N.: Evaluation and classification of the potential carcinogenicity and mutagenicity of chemical biorefractories identified in drinking water. A collaborative study between the NCI, SRI International, Mitre Corporation and the Department of Energy, February, 1981. GPO, 1981-341-132/-3582.

Kelsey, M. I., Hwang, K. -K., Huang, S. -K. and Shaikh, B.: Characterization of microbial metabolites of lithocholic and sulfolithocholic acid by high-performance liquid chromatography. J. Steroid Biochemistry 14: 205-211, 1981.

Proceedings of the First NCI/EPA/NIOSH Collaboratrive Workshop: Progress on Joint Environmental and Occupational Cancer Studies (May 1980). Editors: H. F. Kraybill, I. C. Blackwood and N. B. Freas. National Cancer Institute, Bethesda, Maryland, March 1981. GPO 1981-341-132/3522.

Kelsey, M. I., Kraybill, H. F., Helmes, C. T., Sigman, C. C.: A Data Base of Organic Pollutants that have been evaluated for carcinogenicity and mutagenicity. Proceedings of 1981 International Conference on Residential Solid Fuels, Portland, Oregon, June, 1981. (In press)

Kelsey, M. I., Pienta, R. J.: Transformation of hamster embryo cells by neutral sterols and bile acids. Toxicology Letters (In press).

Kraybill, H. F.: Carcinogenesis of synthetic organic chemicals in drinking water. Journal of the American Water Works Association, Denver, Colorado (In press).

Highlights

From prior years planning and some execution on the part of staff, we were able this year to see some of our plans come to fruition by certain published reports. Our workshop on the collaborative programs of NCI/EPA and NCI/NIOSH resulted in a rather comprehensive and creditable Proceedings on Environmental and Occupational Carcinogenesis. For those reviewing the proceedings it must be evident that this cooperative venture is achieving high quality research and technical developments that may not have been achieved otherwise. It is hoped that our efforts will be equally productive next year. A workshop was held in September 1981, similar in scope and direction to the one held in May 1980. The annual report on the NCI/EPA collaborative program has been improved in format and scope. This resource document is also a visible instrument of accomplishment.

This year considerable progress was made in the development of data bases on carcinogens in drugs and cosmetics. Appropriate liaison has been maintained with FDA and industry in these developments since this work will lead to publication of monographs, which will be useful reference documents or handbooks. The development of a data base on atmospheric carcinogens is leading now to publication of papers and, again, a reference document which will be distributed on carcinogenic air pollutants. After publishing several journal articles on water carcinogens, this year a synthesis of all this material evolved into a report entitled "Biorefractories in Water: Carcinogens, Mutagens and Promoters." These documents have wide utility such as their usefulness in chemical selection and as reference material for IARC monographs and position papers on this subject.

There has been a shift in emphasis in the utilization of one of our contract projects which was focalized predominantly on chemical selection for carcinogenesis bioassay. This year more effort was placed on development of research documents comprising information and data on environmental carcinogens needed by the Division and this office. The support function for chemical selection was markedly reduced.

The Division, represented through this office, sponsored a workshop on Exposures and Metabolism, a work phase of the Task Force on Environmental Cancer and Heart and Lung Disease (DHHS and EPA); a work project required in the form of an annual report to U. S. Congress. The scientific input to this report, by a subcommittee sponsored by NCI containing about 72 recommendations for initiatives and strategies in this area, was considered a sophisticated and excellent report. NCI deserves much credit for taking the leadership in assisting other agencies and the lead agency EPA attain the goals set forth by a congressional committee.

As in the past year, this office furnished a report on chemicals specified for testing and research that will be submitted to Congress in response to legislation enacted in 1978 under the Biomedical Research and Training Act (Maguire Amendment).

Two in vitro mutagenicity contracts were awarded to serve as resources to the chemical selection process and the intramural research activities of the Division. The Salmonella-Microsome Assay and the Mouse Lymphoma Assay will each be used to test up to 75 compounds per year, with and without metabolic activation. Up to 25 compounds will be retested in the event that the initial studies result in equivocal findings.

CONTRACT NARRATIVES

OFFICE OF THE SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

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Technical Resources, Inc. N01-CP-15761	Survey of Compounds Which Have Been Tested for Carcinogenicity (PHS-149), Supplements for 1974, 1975, 1976, 1977, 1979, 1980.	50
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ARTHUR D. LITTLE, INC (N01-CP-85677)

Title: Evaluation of the Transformation Assay Using C3H 10T½ Cells for Use in Screening Chemicals for Carcinogenic Potential

Contractor's Project Director: Dr. Andrew Sivak

Project Officer: Dr. Thomas P. Cameron

Objectives: The objective of this project is to evaluate and determine the usefulness and reliability of this assay for inclusion in a battery of short-term assays. A secondary objective is the incorporation of an intact rat hepatocyte metabolic activation system as a routine protocol ingredient to detect transformation potential of chemicals, otherwise likely to be missed, that are not direct acting.

Major Findings: Transformation experiments have been conducted with some 25 coded chemical samples supplied by the NCI and tested in the absence of an exogenous metabolic activation system. Preliminary studies have been conducted to determine the feasibility of using rat hepatocytes for metabolic activation; evidence suggests that they are effective for some but not all classes of chemicals. Evaluation of cell populations derived from a number of Type III foci were also carried out. Cells at passages two and eight did not grow in soft agarose but did grow at passage 15. None produced tumors in irradiated weanling C3H mice.

Significance to Biomedical Research Program of the Institute: This contract effort, checked by a parallel contract, will serve to sharply define the predictive value of this assay. In the event that this assay can be shown to reliably indicate the transformation potential of these coded compounds whose carcinogenic activity has been previously verified by long-term rodent bioassays, it will be a major step forward in substituting an inexpensive short-term test for the very expensive and time-consuming lifetime rodent studies heretofore the mainstay of our carcinogen detection efforts.

Proposed Course: A Justification for Noncompetitive Procurement of this contract for a final two years has been submitted. This would permit a complete and definitive system validation by testing an additional 50 coded samples without activation. In addition, all negatives would be retested with the intact rat hepatocyte system and that activation system finalized into the standard protocol.

Date Contract Initiated: September 30, 1978

Current Annual Level: $230,000

CENTER FOR DISEASE CONTROL (Y01-CP-60215)

Title: Human Health Consequences of Polybrominated Biphenyls (PBBs) Contamination of Farms in Michigan

Contractor's Project Director: Dr. Matthew Zack

Project Officer (NCI): Dr. Thomas C. Cameron

Objective: As a consequence of the distribution of animal feeds contaminated with PBB, the inhabitants of at least 412 Michigan farms, as well as a wide surrounding belt of consumers, were accidentally exposed to that potentially threatening compound. This activity defines and recruits large cohorts of individuals (of several degrees of exposure) and will engage in a long-term follow-up primarily with respect to the incidence of cancer.

Major Findings: The major effort to date has been the development of cohorts. Two blood samples have been taken from all PBB exposed individuals and analyzed for this compound, as well as polychlorinated biphenyls (PCBs) which is of equal interest scientifically and provides gratuitous information. Certain high risk groups (high blood level individuals engaged in the production of the PBB compound and children born during or after the incident) continue to be monitored closely. The initial scientific impression that there were little or no immediate or acute effects on the subject population appears to have been verified.

Significance to Biomedical Research and Program of the Institute: This is a unique situation in which a large group of people had a known exposure to a potential carcinogen under sharply defined circumstances of geography and time. Unfortunate subsequent rises in cancer incidence, if they occur, can thus be charted in this prospective study and correlated with modes and amount of exposure. Major scientific obervations on this particular compound and structurally allied compounds can be developed.

Proposed Course: Cohorts having been defined and recruited, the major effort for the foreseeable future is to maintain their integrity for the long-term determinations. Deaths, illness, pregnancies, etc., will be carefully monitored. It is anticipated that the scaling down of effort will continue to some degree because of the completion of major tasks.

Date Contract Initiated: April, 1976

Current Annual Level: $165,000

EG&G MASON RESEARCH INSTITUTE (N01-CP-15739)

Title: In Vitro Evaluation of Chemical Candidates for In Vivo Testing

Contractor's Project Director: Dr. Paul Kirby

Project Officer: Dr. Thomas P. Cameron

Objectives: The testing of chemical compounds in the mouse lymphoma in vitro assay provides valuable information that is utilized in the selection of candidates nominated for chronic in vivo testing by the National Toxicology Program. In addition, support is provided to intramural scientists who require mutagenicity information not otherwise available, as an adjunct to their own investigations.

Major Findings: The testing of compounds has been initiated, and the first reports of positive or negative mutagenicity have been received.

Significance to Biomedical Research Program of the Institute: This contract will serve to eliminate "gaps of knowledge" concerning the biological effects of specific chemicals. This will facilitate the accurate selection of candidates for bioassay and insure the optimal employment of that prolonged and extremely expensive test system. The same information supplied to intramural scientists will supplement their investigations in chemical carcinogenesis without diverting staff time and scarce laboratory space to setting up in vitro systems that would be used only intermittently.

Proposed Course: It is intended that this contract will be executed for its authorized three-year period. At yearly intervals, the results of this contract will be evaluated against those of another contract (Salmonella/typhimurium) testing essentially the same compounds to obtain the additional bonus of comparing two assay systems.

Date Contract Initiated: March 20, 1981

Current Annual Level: $340,527

ENERGY, DEPARTMENT OF (Subcontractor: Mitre Corporation) Y01-CP-80202

Title: Interagency Agreement on Study of the Human Health Significance of the Potentially Carcinogenic Energy-Related Organic and Inorganic Contaminants in Water

Contractor's Project Director (Mitre): Dr. Robert Clerman

Project Officer (NCI): Dr. H. F. Kraybill

Project Officer (DOE): Dr. Nathaniel Barr

Objectives: To develop a state of the art report on bioassay, monitoring, identification and classification of energy and environmental chemicals that are carcinogens and/or mutagens. This year more emphasis was placed on exposure levels of carcinogens in water. Evaluation of epidemiological studies on water contaminants and adverse effects in aquatics and mammalian species including carcinogenic effects from these aquatic exposures.

Major Findings: The first year of effort produced a comprehensive report entitled "An Assessment of Potentially Carcinogenic Energy Related Contaminants in Water;" a 381 page report. This report has been widely used and became a comprehensive reference on aquatic pollutants with a select list of carcinogens and mutagens in drinking water, surface water and ground water. This year more emphasis was placed on exposures for carcinogens and mutagens in drinking water and a risk assessment made on these contaminants.

Significance to Biomedical Research and Program of the Institute: In a program on environmental cancer, the question is raised about the carcinogenic agents in the environment, their occurrence and level of exposure, and how the presence of such carcinogenic contaminants may impact on man. This work, and the reports produced, address this problem and identify and classify these chemicals and the magnitude of such exposures. It also delineates what extent these new energy technologies have on this contaminant problem and what risk may be imparted to man and the aquatic environment by pollution from these chemicals.

Proposed Course: This project was extended beyond the original one-year plan to concentrate on more data on exposures and risk from such exposures from carcinogenic/-mutagenic contaminants in water. An annual report will be issued sometime in August 1981.

Date Contract Initiated: March, 1978

Current Annual Level: $125,000

ENERGY, DEPARTMENT OF (Subcontractor: Associated Universities, Brookhaven National Laboratory) N01-CP-90202

Title: Project to Coordinate Information and Technological Transfer in Environmental Mutagens and Carcinogens

Contractor's Project Director: Dr. Alexander Hollaender

Project Officer: Dr. H. F. Kraybill

Objectives: The National Cancer Institute, in collaboration with EPA, NIEHS and DOE, supports training centers for instruction of national and international scientists, through leadership of Dr. Hollaender, on procedures and information and data bases in the area of mutagenesis and carcinogenesis.

Major Findings: As a follow-up of last year, more workshops were held to provide orientation at several institutions in the USA and abroad. This indoctrination provides needed skills for laboratory workers and, as a result, prepared a cadre of trained experts to interpret these genetic toxicology tests which are important in assessment of health effects.

Significance to Biomedical Research Program of the Institute: This training program prepares a cadre of trained professionals in this specialized area which can relate to program managers and science administrators at NCI. This has both a direct and indirect spinoff to contract and grants projects supported by NCI.

Proposed Course: While this project has developed a national and international resource via professionals trained in this area, with limitation of funds, NCI decided to extend project into 1981 with the residue of funds left over but no continuation or additional funds were anticipated and hence this project will terminate.

Date Contract Initiated: April, 1979

Current Annual Level: No funds allocated in FY81

ENVIRONMENTAL PROTECTION AGENCY (Y01-CP-80205 - Office of Toxic Substances and Office of Research and Development

Title: Interagency Agreement for Performance of Collaborative Studies in Area of Environmental Cancer

Contractor's Coordinators or Project Directors:

NCI: Dr. H. F. Kraybill
EPA: Drs. William Farland (OTS) and Wayne Galbraith (ORD)

Objectives: In response to request from OMB and U. S. Congress, NCI entered into a collaborative agreement with EPA to jointly conduct projects on environmental cancer (experimental, epidemiological and information resources). This program was initiated 3 years ago with a total of about 30 projects, half of which are monitored by NCI, the other half by EPA. All projects have project officers and co-project officers (list of projects are given in table). In practice the total funding of $4 million is spent in half with transfer annually of about $2 million to EPA to implement and carry out these projects.

Major Findings: As to information resource, one project has resulted in the annual publication of a comprehensive report on "Chemicals Found in Human Biological Media: A Data Base." This is a data base made available to federal agencies and state health departments. The other projects in the experimental and epidemiology areas have shown some important breakthroughs on the role of environmental chemicals in cancer induction. In one project, a Gulf Breeze, Florida laboratory, in studying tumorigenesis in aquatic animals, has shown that one species, the mullet, handles the carcinogen BAP the same as the rodent.

Significance to Biomedical Research and Program of the Institute: Until this program was mandated, it was not feasible to really emphasize adequately the significance of studying extensively environmental chemicals in various media (air, water, and diet) and how this impacts on human cancer. These projects provide for some visibility in this area. This emphasis provides NCI with some important data on environmental cancer, as well as supporting the mission and program initiatives of EPA in environmental health and regulation and control of environmental insults. Studies on water carcinogens, UV radiation, hexachlorobenzene, asbestos, and other atmospheric carcinogenic contaminants are examples of problems addressed. This program is being followed with much interest by the U. S. Congress.

Proposed Course: Many of the projects are planned for 3-5 years. At our Annual Workshop, suggestions for new areas to be covered were considered. The EPA projects are more slanted to experimental studies, while NCI projects are predominantly epidemiological. An effort will be made to maintain some representative balance and to keep an equal distribution of projects and funding.

Date Contract Initiated: June, 1978

Current Annual Level: $4,000,000 ($2 million to NCI and $2 million to EPA)

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (N01-CP-15751)

Title: Program on Evaluation of Carcinogenic Risk of Chemicals to Man

Contractor's Project Director(s): Drs. John Higginson and Lorenzo Tomatis

Project Officer: Dr. H. F. Kraybill

Objective: The IARC prepares monographs on environmental chemicals relevant to evaluation of carcinogenic risk of these chemicals to man. Implementation of the IARC Monograph Program involves three steps: 1) the collection of data relevant to the assessment of carcinogenic risk of these chemicals to which humans may be exposed, 2) the critical analysis and evaluation of these data by IARC staff and international working groups of experts in chemical carcinogenesis and epidemiology convened in Lyon to make these evaluations, and 3) the editing and preparation of authoritative monographs and distribution of these monographs assembled in volumes. NCI receives 250 copies of these volumes (about 3 times per year) for distribution. Anciliary to this project, another project is supported to collect data on testing of chemicals for carcinogenicity in laboratories around the world including the USA (NCI and NTP). This data is compiled into an Information Survey Bulletin. This Bulletin is prepared and distributed every 18 months.

Major Findings: To date 25 volumes of the monographs have been issued with at least two more (Vols. 26 and 27) this fiscal year. Also expected to be issued this year is Volume 9 of the Information Survey Bulletin. Both of these efforts are indispensable since they are widely used reference sources which are authoritative and quoted by all scientists in this field.

Significance to Biomedical Research and Program of the Institute: As an information and data resource, the IARC Monographs have become an invaluable reference to provide instantaneous evaluations and judgements as to appraisement of carcinogenicity for about 400 chemicals, with the list expanding. The chemicals are selected on the basis of use and structure class. IARC also publishes supplements on methodology and a recent compilation of chemicals in a listing of chemicals carcinogenic to man and experimental animals. The IARC is now publishing monographs on chemical processes such as woodworking, leather working and rubber processing with attention to epidemiological studies. The Information Bulletin is a useful reference source for laboratory workers engaged in chemical carcinogenesis.

Proposed Course: The contractor will continue on this cost-sharing project (USA contributes 60-70%; the remaining funds from member countries) and will convene advisory working groups 3 times a year in connection with monographs preparation. IARC staff will continue to compile data for the Information Survey Bulletin. Consideration was given this year to setting up a cooperative agreement for 3 years rather than the usual contract which was extended only one year as a JNCP contract (12-80 to 12-81).

Date Contract Initiated: May, 1974

Current Annual Level: $496,467

LAWRENCE JOHNSON ASSOCIATES (N01-CP-15746)

Title: Support of Second Workshop on NCI/EPA and NCI/NIOSH Collaborative Programs on Environmental and Occupational Carcinogenesis

Contractor's Project Director: Ms. Gana Browning

Project Officer: Ms. I. C. Blackwood

Objectives: The objective of the Second Annual NCI/EPA/NIOSH Workshop on Collaborative Projects on Environmental and Occupational Cancer is to report on progress and decide on future direction of projects carried out under two interagency agreements between the National Cancer Institute and the Environmental Protection Agency and the National Institute for Occupational Safety and Health, respectively. The purpose of this contract is to provide conference support and assistance in editing the proceedings resulting from this workshop.

Major Findings: Since the workshop will take place in September of this year, no major findings are reportable yet.

Significance to Biomedical Research and Program of the Institute: This workshop provides a forum for the exchange of findings and information developed from the various projects sponsored under the two interagency agreements on environmental and occupational cancer. The results of these studies have potential applicability for protecting not only workers but the general population from exposure to carcinogens both in the workplace and the environment.

Proposed Course: Since the first workshop, held last year, proved to be quite successful, this year's effort will proceed along similar lines with the contractor assisting the National Cancer Institute with the administrative planning and management of the meeting and the editing of the proceedings. To make the information discussed during this workshop available to a wider segment of the federal government and the scientific community at large, the meeting will be open to the public.

Date Contract Initiated: April, 1981

Current Annual Level: $50,000 (includes editing of proceedings)

MICROBIOLOGICAL ASSOCIATES (N01-CP-85617)

Title: Evaluation of the Transformation Assay Using C3H 10T½ Cells for Use in Screening Chemicals for Carcinogenic Potential

Contractor's Project Director: Dr. Leonard M. Schechtman

Project Officer: Dr. Thomas P. Cameron

Objectives: The objective of this project is to evaluate and determine the usefulness and reliability of this assay for inclusion in a battery of short-term assays. A secondary objective is the incorporation of an S-9 metabolic activation system as a routine protocol ingredient to detect transformation potential of chemicals, otherwise likely to be missed, that are not direct-acting.

Major Findings: Tranformation experiments have been conducted with some 35 coded chemical samples supplied by the NCI and tested in the absence of an exogenous metabolic activation system. Model compounds known to be negative have been run in the presence of the S9 preparation. The S-9 preparations have been thoroughly characterized as to their content, ability to metabolize, and inherent cytotoxic effects on the 10½ cells.

Significance to Biomedical Research Program of the Institute: This contract effort, checked by a parallel contract, will serve to sharply define the predictive value of this assay. In the event that this assay can be shown to reliably indicate the transformation potential of these coded compounds whose carcinogenic activity has been previously verified by long-term rodent bioassays, it will be a major step forward in substituting an inexpensive short-term test for the very expensive and time-consuming lifetime rodent studies heretofore the mainstay of our carcinogen detection efforts.

Proposed Course: A Justification for NonCompetitive Procurement of this contract for a final two years has been submitted. This would permit a complete and definitive system validation by testing an additional 50 coded samples without activation. In addition, all negatives would be retested with the S-9 system and the incorporation of the S-9 system into the protocol will be finalized.

Date Contract Initiated: September 30, 1978

Current Annual Level: $219,000

NATIONAL INSTITUTE OF OCCUPATIONAL SAFETY AND HEALTH (Y01-CP-60505)

Title: Conduct of Research on Occupational Carcinogenesis

Contractor's Project Director: Dr. Kenneth Bridbord

Project Officer (NCI): Dr. Thomas P. Cameron

Objective: This agreement was originated as a Congressionally mandated effort to integrate and thereby enhance the activities of both the National Cancer Institute and the National Institute for Occupational Safety and Health in investigating chemical-related occupational cancer.

Major Findings: To date some 71 distinct projects have been initiated, of which approximately 25 are in progress. Most of the projects were reported on in a combined NCI/EPA/NIOSH Collaborative Workshop held in September 1981.

Significance to Biomedical Research and Program of the Institute: The merging of the unique capabilities of each organization into a concerted investigation of a variety of industrial situations promises an acceleration of the response to the question of the quantification of occupationally-caused cancer. In that quest, answers to the many questions concerning acute toxicity, safe ambient levels, etc., which are more germane to the NIOSH mission, will accrue. NIOSH contributes the capabilities of freely entering industrial operations, as well as considerable experience in surveying potentially dangerous situations therein.

Proposed Course: The on-going projects shall be continued as dictated by their objectives and workscopes. New projects shall be begun as a result of initiation by the staff of either organization and relevance and technical review by a combined ad hoc committee of the senior staff members of both institutes. Obligation of funds by the NCI for the ensuing fiscal year shall in no case exceed the sum of four million dollars.

Date Contract Initiated: September, 1974

Current Annual Level: $4,000,000

RESEARCH TRIANGLE INSTITUTE (N01-CP-15740)

Title: In Vitro Evaluation of Chemical Candidates for In Vivo Testing

Contractor's Project Director: Mr. Thomas Hughes

Project Officer: Dr. Thomas P. Cameron

Objectives: The testing of chemical compounds in the Salmonella/typhimurium in vitro assay provides valuable information that is utilized in the selection of candidates nominated for chronic in vivo testing by the National Toxicology Program. In addition, support is provided to intramural scientists who require mutagenicity information not otherwise available, as an adjunct to their own investigations.

Major Findings: The testing of compounds has been initiated, and the first reports of positive or negative mutagenicity have been received.

Significance to Biomedical Research Program of the Institute: This contract will serve to eliminate "gaps of knowledge" concerning the biological effects of specific chemicals. This will facilitate the accurate selection of candidates for bioassay and insure the optimal employment of that prolonged and extremely expensive test system. The same information supplied to intramural scientists will supplement their investigations in chemical carcinogenesis without diverting staff time and scarce laboratory space to setting up in vitro systems that would be used only intermittently.

Proposed Course: It is intended that this contract will be executed for its authorized three-year period. At yearly intervals, the results of this contract will be evaluated against those of another contract (mouse lymphoma) testing essentially the same compounds to obtain the additional bonus of comparing two assay systems.

Date Contract Initiated: March 31, 1981

Current Annual Level: $115,000

SRI, INTERNATIONAL (N01-CP-05711) BOA

Title: Carcinogenicity of Cosmetics

Contractor's Project Director: Dr. Tucker Helmes

Project Officer (NCI): Dr. Morris I. Kelsey

Objectives: This project provides resource information on the chemical and biological activity of cosmetic ingredients.

Major Findings: The contractor is preparing 12-13 IARC-style monographs on currently used cosmetic ingredients that were selected by an outside steering committee.

Significance to Biomedical Research and Program of the Institute: The bound report of monographs dealing with the potential carcinogenicity of cosmetic ingredients will be an important resource to the Office of Environmental Cancer in responding to inquiries from the Division, Institute, and other interested parties. Such information is crucial to the mission of our Office in gathering data on carcinogens present in various environmental media that result in multiple chemical insults to which humans are exposed.

Proposed Course: The contract is now operating under a two-month, no-cost extension. A steering committee is currently reviewing proposed task descriptions prepared by SRI in order to determine if they should be awarded an extension of the current contract. This extension is desired by NCI to generate additional monograph candidates from cosmetic ingredients, fragrances and possibly contaminants. If SRI does not satisfy the committee with respect to their technical approach, then the BOA will be recompeted amongst the other three offerors (Tracor-Jitco, Inc., Syracuse Research Corporation and Systems Application, Inc.).

Date Contract Initiated: September, 1980

Current Annual Level: $77,400

SRI INTERNATIONAL (N01-CP-95607)

Title: Resource to Support the Chemical, Economic and Biological Needs of NCI/DCCP and to Provide Support to the International Agency for Research on Cancer (IARC). The support includes information resource data on environmental carcinogens.

Contractor's Project Director(s): Dr. Tucker Helmes and Ms. Mary Doeltz.

Project Officer(s): Dr. H. F. Kraybill and Dr. M. I. Kelsey (Assistant Project Officer).

Objectives: This project is an important information and data resource. The project has five tasks as follows: 1) Support to NCI Chemical Selection Working Group; 2) Support to IARC for monograph preparation; 3) Updating and expanding data base on exposures and data base on mutagens, carcinogens, co-carcinogens and promoters; 4) Data processing services and support; and 5) Overall support to DCCP and Office of Environmental Cancer on resource research documents on environmental cancer. In addition, there may be a task added to give support to National Library of Medicine.

Major Findings: This project is in the second year of a planned five year project. It provides information and data on summary sheets which are used in NCI's chemical selection process for chemicals to be tested in the National Toxicology Program (NTP). The contractor furnishes production, exposure and chemical data on chemicals that are entered into the IARC monographs. SRI has completed a series of Class Studies (use and structure class), which will be published, that serve as a basis for chemical selection. SRI has prepared reports on carcinogens in air and water which are utilized in the carcinogenesis program and as a basis for selection of environmental chemicals to be tested. The contract project has shifted in emphasis for a predominant resource to assist CSWG to perform special studies and resource needs as to data on environmental carcinogens and publication of compendiums on carcinogens that serve as resource/reference documents.

Proposed Course: The SRI project will continue to support needs on chemical selection process but at reduced effort of 25 summary sheets per year (about one-half production of last year). Under Task V, more subtasks have been outlined for preparation of special reports on environmental carcinogens that can be used by NCI and DCCP as authoritative reference sources.

Significance to Biomedical Research and Program of the Institute: This project continues to be one of the most important resources for NCI, NTP and other agencies for information on environmental chemicals. The reference documents prepared have been most useful to NCI in the carcinogenesis testing and research programs. The resource is used in preparation of lists of chemicals for carcinogenesis testing and research under the Biomedical Research and Training Act.

Date Contract Initiated: November, 1979

Current Annual Level: $872,100

TECHNICAL RESOURCES, INC. (N01-CP-15761)

Title: Survey of Compounds Which Have Been Tested For Carcinogenicity (PHS-149); Supplements for 1974, 1975, 1976, 1977, 1979 and 1980.

Contractor's Project Director: Mr. Anthony Lee

Project Officer: Dr. H. F. Kraybill

Objectives: This project provides a resource for preparation of PHS-149, Survey of Compounds for Carcinogenicity for the missing years - 1974-1977, 1979 and 1980. This survey is made from literature worldwide.

Major Findings: Since there was a lapse in publication of this widely used resource report, a contractor produced a report for 1978. The current contract, instituted in April 1981, will produce, over a 3 year period, volumes for 1974-77, 1979 and 1980. In the future a contract will be secured to keep this publication current.

Significance to Biomedical Research and Program of the Institute: This report will provide the information resource for staff at NCI, academia and other research organizations setting forth data gleaned from the world literature on chemicals bioassayed (about 650 journals worldwide) for carcinogenicity. This report, first published in 1951, has come to be recognized as an indispensable standard reference source for workers in the field of carcinogenesis. NCI distributes these surveys and the Superintendent of Documents (GPO) retains some for sale.

Proposed Course: The contractor will work on this project over three years, producing two camera-ready volumes for each year. GPO will handle the printing. While this effort will make us current, by the time these 6 volumes are published, we will need to consider publication for 1981, 1982, 1983, and 1984.

Date Contract Initiated: April, 1981

Current Annual Level: $253,519 (two volumes)

TRACOR-JITCO, INC. (N01-CP-05633-01) BOA

Title: Carcinogenicity of Drugs and Medical Procedures

Contractor's Project Director: Dr. Harold Seifried

Project Officer (NCI): Dr. Morris I. Kelsey

Objectives: The project provides resource information on the chemical and biological activity of drugs and medical procedures.

Major Findings: The contractor has prepared its draft report "Genotoxic Assessment of Selected Drugs and Medical Procedures," which contains monographs on 109 drugs and 15 medical procedures. This document will now be reviewed by various outside committees and will be published as a government report.

Significance to Biomedical Research and Program of the Institute: The bound report of monographs dealing with the potential carcinogenicity of drugs and medical procedures will be an important resource to the Office of Environmental Cancer in responding to inquiries from the Division, Institute and other interested parties. Such information is crucial to the mission of our Office in gathering data on carcinogens present in various environmental media that result in multiple chemical insults to which humans are exposed.

Proposed Course: The contractor will utilize a new series of data bases to add the important exposure information to the above mentioned monographs. This contract will be extended to utilize the new data bases for additional candidates for monographs on drugs and medical procedures.

Date Contract Initiated: May, 1981

Current Level of Funding: $167,417

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04548-09 OD

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Registry of Experimental Cancers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Harold L. Stewart	NIH Scientist Emeritus	DCCP	NCI
	Bernard Sass	Veterinary Medical Officer	DCCP	NCI
	Margaret K. Deringer	Biologist	DCCP	NCI
	Margaret D. Miller	Guest Worker	DCCP	NCI
	Cornelia Hock-Ligeti	Guest Worker	DCCP	NCI
	Carel F. Hollander	Consultant	DCCP	NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Division of Cancer Cause & Prevention, Office of Director

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The objectives of the Registry of Experimental Cancers are 1) the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents) and 2) the use of such information for research and educational purposes.
2. The Registry has acquired a total of 1,979 (96 since the 1980 report) single or group accessions from investigators outside the NCI. Approximately 55,000 (1,800 since the 1980 report) records have been prepared for coding and coded.
3. Forty-one investigators have come to the Registry for study and consultation on single or multiple visits. Three foreign investigators were guests at the Registry, 2 for 1 day each and 1 for 3 days.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1) The storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents), and 2) the use of such for research and educational purposes.

Methods Employed: The methods employed in the work of the Registry involve the selection of protocols, pathologic material, including histologic slides, paraffin blocks, and illustrations in the form of lantern slides, gross photographs, and photomicrographs in black and white and in color. The work of the Registry also includes the collection of records of experiments, reprints and references on this material. The Registry of Experimental Cancers possesses a large collection of spontaneous and induced cancers and other lesions. The pertinent information on the collection is indexed. Many of the data have been prepared for and entered into the computer. The Registry accesses material from investigators at NCI, other institutes of NIH, other governmental agencies, industrial laboratories, and universities here and abroad. A total of 1,979 single or group accessions from investigators outside of NIH have been processed since 1971. The Registry has prepared and is preparing Study Sets of slides, with explanatory notes, relating to particular cancers of rodents.

The Registry has Study Sets of slides on "Hematopoietic and Lymphoreticular Neoplasms," "Induced and Spontaneous Tumors of Small Intestine and Peritoneum in Mice," "Tumors and Nonneoplastic Proliferative Lesions of the Lungs of Mice," "Induced Tumors of the Liver in Rats," "Mammary Tumors in Mice," "Pulmonary Metastasis in Mice," "Neoplastic and Nonneoplastic Lesions of the Lymphoreticular Tissue in Mice," and "Neoplasms and Other Lesions of Praomys (Mastomys) Natalensis." The last three Study Sets have been completed since the 1980 Annual Report. These Study Sets, with descriptive material, are loaned to investigators who request them. Thirteen loans up to two months have been made this year.

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 41 individuals since the 1980 report. Three foreign investigators have been guests at the Registry, two for one day and one for three days.

Major Findings: The functions (outlined in objectives) of the Registry in the field of cancer research are such that there will be no major findings to report.

Significance to Biomedical Research and the Program of the Institute: The availability of the wealth of material possessed by the Registry advances the knowledge of spontaneous and induced disease processes in animals. It is a national and international resource.

The existence of the Registry will contribute to the standardization of nomenclature of cancers and other lesions in laboratory rodents. Slides and protocols from the Registry are used to illustrate and describe lesions discussed at the weekly slide conferences held in Room 407 of the Del Ray Building.

The members of the Registry serve as consultants in the monitoring of pathology from laboratories of NCI, of other institutes of NIH and elsewhere.

The Subcommittee on Rat Liver Tumors, appointed by the Institute of Laboratory Animal Resources, NRC, NAS, carried out its work at the Registry of Experimental Cancers where it met one or two days each month over a period of 18 months. The members of the Subcommittee studied the pathologic material in the Registry's Study Set on Rat Liver Tumors and other pathologic materials accessed at the Registry. The publication of the report entitled "Histologic Typing of Liver Tumors of the Rat" appeared in January of 1980 (J. Natl Cancer Inst. 64:177-206, 1980). During the period from the date of publication until April 2, 1981, the Registry has received 5,081 requests for reprints. This histologic classification and typing of rat liver tumors is calculated to promote uniformity of diagnoses from one laboratory to another in this country.

The Director General of the World Health Organization designated the Registry of Experimental Cancers as the WHO Collaborating Centre for Reference on Tumours of Laboratory Animals on October 26, 1976. This is the only such registry in the world to be so designated by the WHO. This will expand communications between scientists of this country and those of other countries, now numbering 153, which are members of WHO.

Proposed Course : The Registry will continue and expand all of its activities (already set forth in this report).

Publications:

Hoch-Ligeti, C., Congdon, C. C., Deringer, M. K., Strandberg, J. D. and Stewart, H. L.: Hemangiopericytoma and other tumors of urinary tract of guinea pigs. Toxicol. Pathol. 8: 1-8, 1980.

Sass, B. and Montali, R. J.: Spontaneous fibro-osseous lesions in aging female mice. Lab. Anim. Sci. 30: 907-909, 1980.

Stewart, H. L.: A central repository for cancers of captive wild animals and birds. In Montali, R. J. and Migaki, G. (Eds.): The Comparative Pathology of Zoo Animals. Washington, Smithsonian Institution Press, 1980, pp. 543-547.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05072-02 OAD

PERIOD COVERED
September 30, 1980 - October 1, 1981

TITLE OF PROJECT (80 characters or less)

Feline Sarcoma and Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	C. J. Sherr	Head, Viral Pathology Section	OAD, NCI
	J. Even	Visiting Fellow	OAD, NCI
	S. J. Anderson	Staff Fellow	OAD, NCI

COOPERATING UNITS (if any)

S.K. Ruscetti	Staff Fellow,	LTVG, NCI
M. Barbacid	Expert	LCMB, NCI
F. Galibert	Professor,	Hopital St. Louis, Paris, France

LAB/BRANCH
Office of the Associate Director

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, MD, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	3	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The DNA proviruses of the Snyder-Theilen (ST), Gardner-Arnstein (GA) and McDonough (M) strains of feline sarcoma virus (FeSV) as well as that of feline leukemia virus (FeLV, subgroup B) were molecularly cloned in bacteriophage vectors. Each sarcoma virus is a genetic recombinant containing portions of the FeLV genome linked to transforming gene sequences (onc) transduced from normal cat cellular DNA. The viral onc elements of ST- and GA-FeSV (v-fes) share homologous sequences representing similar portions (exons) of a segmented cat cellular gene (c-fes). The v-fes sequences specify a tyrosine-specific protein kinase (PK) activity. Transformation-defective and nontumorigenic mutants of ST-FeSV encode products lacking PK activity showing that PK is required for morphological transformation. The amino acid sequence of the v-fes coded kinase has been deduced by nucleotide sequence analysis. The transforming gene sequences of M-FeSV represent a distinct onc element showing that FeLV can transduce at least two cellular transforming genes. Both feline onc genes are evolutionarily conserved and are homologous to sequences in the cellular DNA of other avian and mammalian species, including man.

Objectives:

(i) To study the structure and genetic organization of feline sarcoma viruses. (ii) To identify the genetic information required for FeSV-induced cellular transformation. (iii) To elucidate the role of FeLV-derived sequences in regulating the expression of the recombined, transduced onc elements acquired from normal cat cells. (iv) To characterize the cellular gene sequences homologous to viral transforming gene sequences. (v) To identify and characterize FeSV encoded proteins which are required for morphological transformation.

Methods Employed:

Extrachromosomal DNA was chromatographically purified from host cells infected with ST-FeSV (FeLV) and was used as a substrate for restriction enzyme analysis using the Southern blotting procedure. Eco RI-digested viral DNA was ligated into a bacteriophage vector and recombinant phages were isolated using plaque blotting techniques. High molecular weight cellular DNA was extracted from cells nonproductively transformed with either GA- or M-FeSV. After Eco RI restriction, the proviral-containing DNA fragments were partially purified by centrifugation and electrophoretic techniques and ligated into bacteriophage vectors. Recombinant phages containing FeLV, ST-FeSV, GA-FeSV, and M-FeSV proviral DNA sequences were characterized by heteroduplexing, R-looping with viral RNA, and restriction enzyme analyses. The complete nucleotide sequence of the cloned ST-FeSV DNA was obtained using a combination of the Maxam-Gilbert and Sanger sequencing methods.

Segments of the ST- and GA-FeSV proviruses corresponding to different portions of the viral genomes were subcloned in the plasmid pBR322. Subclones corresponding to regions of the viral onc sequences (designated v-fes) were used to map homologous cat cellular sequences (designated c-fes) by the Southern blotting technique. Plasmids containing the complete GA-FeSV provirus or various subgenomic fragments were transfected onto NIH/3T3 cells to assay for transforming activity. Genetically engineered viral mutants and interstrain recombinants were constructed and similarly assayed for transforming functions.

Antisera to viral gene products were prepared in rats inoculated with rat cells nonproductively transformed by the different FeSV strains. Sera from tumor bearing animals were absorbed with FeLV proteins and screened for antibodies directed to onc gene coded products. The antisera were used to precipitate FeSV polyproteins from transformed mink, rat, mouse, or cat cells metabolically labeled with different precursors (^{3}H-leucine, ^{3}H-mannose, ^{3}H-galactose, ^{35}S-methionine, ^{32}P-phosphoric acid), and the immune precipitates were solubilized and electrophoretically analyzed on polyacrylamide gels under denaturing conditions. Unlabeled immune complexes were incubated *in vitro* with ^{32}P-labeled ATP and tested for the presence of protein kinase activity by similar methods. Phosphoaminoacid analyses were performed by two dimensional electrophoretic and chromatographic separation of partial acid hydrolysates in the presence of phosphoserine, phosphothreonine, and phosphotyrosine standards.

Viral mutants isolated from cells following infection at a low multiplicity were virologically cloned under conditions favoring single virus - single cell interactions. Cells containing transformation-defective (td) mutants of ST-FeSV were characterized by Southern blotting of proviral DNA; liquid hybridization of viral RNA (C_rt analysis), solid phase hybridization of polyadenylated mRNA ("Northern blotting"), radioimmunoassays for viral proteins (p15, p12, p30), specific antibody precipitation of metabolically labeled products, and *in vitro* protein kinase assay. Similar techniques were also used to study transformed single cell clones, particularly those derived by DNA transfection.

Major Findings:

ST-FeSV: DNA species of 8.5 and 5.0 kilobase pairs (kb) in length were shown to represent the linear, unintegrated intermediates of ST-FeLV and FeSV, respectively, by the following criteria: (i) Transfection of DNA fractions containing 8.5 or 5.0 kb viral DNA showed that the 5.0 kb form induced foci in mink or mouse cells whereas the 8.5 kb species gave rise to nontransforming, replicating virus in mink or cat cells. (ii) Transcripts complementary to ST-FeSV v-*fes* sequences hybridized only to the 5.0 kb species while cDNA representing sequences unique to the leukemia virus annealed only to 8.5 kb DNA. (iii) The restriction endonuclease Eco RI which recognizes sites at both termini of 5.0 kb DNA and generates an internal 4.3 kb fragment was shown to excise a proviral DNA-containing fragment of corresponding length from the DNA of cells nonproductively transformed by ST-FeSV. Restriction endonuclease sites were mapped within FeSV and FeLV DNA, and three enzymes were identified which failed to cleave the ST-FeSV provirus. Using DNA transcripts complementary to different portions of the FeSV genome, v-*fes* sequences were positioned within 2.0 - 3.4 kb from the 5' end of the DNA provirus.

ST-FeSV and FeLV DNA intermediates were cloned in a prokaryotic vector and propagated in a bacterial host. Recombinant phages were characterized by restriction endonuclease mapping, heteroduplexing, and R-looping with viral RNA. In agreement with the nucleic acid hybridization experiments, v-*fes* sequences were localized within 1.9 - 3.4 kb from the 5' end of the cloned ST-FeSV provirus. The FeLV-derived sequences at the 5' end of the FeSV genome were shown to be derived from the FeLV long terminally redundant (LTR) sequences and a portion of the viral *gag* gene. Sequences 3' to v-*fes* originated from discontiguous regions of the FeLV *env* gene (about 0.6 kb) and the extreme 3' end of viral RNA (about 0.5 kb).

The ST-FeSV genome was shown to encode a high molecular weight polyprotein containing antigens related to the FeLV *gag* gene precursor as well as unique antigenic determinants (designated "x") unrelated to those of FeLV. The order of polypeptides in the *gag* gene precursor is NH_2-p15-p12-p30-p10-COOH; by contrast, only p15, p12, and a portion of p30 are represented in the 85,000 dalton gag-x polyprotein (P85) of ST-FeSV. This implies that the x-portion (about 50,000 daltons) is encoded by the v-*fes* sequences contiguous to *gag*. Using an *in vitro* assay, the ST-FeSV P85 product was shown to exhibit an associated tyrosine-specific protein kinase (PK) activity. In addition, ST-FeSV P85 molecules labeled with ^{32}P-phosphoric acid *in vivo* were themselves found to be phosphorylated in tyrosine. Thus, the polyprotein molecules not only possess an associated PK activity but also serve as substrates for analogous enzymes. Transformation-defective (td) mutants of ST-FeSV were found to synthesize gag-x polyproteins indistinguishable in size and antigenic complexity from wild type P85 molecules. However, the td-ST-FeSV polyproteins lacked detectable kinase activity. These data provided the first genetic evidence that v-fes sequences specify molecules with PK activity.

Several distinguishable forms of P85 were identified in cells transformed by ST-FeSV. The predominant species was readily labeled with ^{32}P-phosphoric acid in vivo and demonstrated associated PK activity. However, a glycosylated form of the polyprotein was also detected following metabolic labeling with ^{3}H-mannose or ^{3}H-galactose. The glycosylated form had a greater apparent molecular weight than the major phosphorylated species and appeared to be less phosphorylated in vivo.

Nucleotide sequencing analysis of ST-FeSV DNA has confirmed and extended the previous findings. In particular: (i) The ST-FeSV polyprotein contains all of p15, p12, and a portion of p30 fused to v-fes sequences of approximately 1.3 kb in length. The complete amino acid sequence of the polyprotein has been deduced. (ii) A leader sequence encoding 72 amino acids (aminoterminus = methionine) precedes the p15 coding region and may specify a signal sequence for intracisternal transport (and subsequent glycosylation). A splice recipient site is localized within this region suggesting that nonglycosylated P85 molecules are encoded by spliced mRNA molecules (aminoterminus = methionine in p15). (iii) The sequence of p15 shows that this region contains a site for glycosylation; p12 has an extremely unusual composition with many acidic residues and 31% proline. (iv) The carboxyterminal end of P85 is homologous to the pp60 src protein encoded by Rous sarcoma virus and, to a lesser extent, to the putative transforming protein of Moloney sarcoma virus deduced by sequence analysis.

The major portions of the ST-FeSV v-fes sequence were subcloned into the plasmid pBR322. The subcloned segments included two Pst I fragments (500 and 475 bases) representing contiguous sequences within v-fes and corresponding to about 75% of the viral onc element. The subcloned sequences were labeled by nick translation and used to map homologous sequences in cat cellular DNA using the Southern blotting technique. The complexity of homologous cellular sequences (c-fes) was found to be much greater than that of the viral sequences used as probe (4.2 versus 0.98 kb, respectively). Restriction enzyme analyses showed that c-fes contained a minimum of three sets of intervening sequences which were not transduced in the viral genome. The segmented nature of c-fes suggests that spliced RNA transcripts serve as intermediates in the formation of sarcoma viral genomes so that only the exon elements are incorporated into transforming viral recombinants.

GA-FeSV: The cellular DNAs of several mink cell clones nonproductively transformed by GA-FeSV were cleaved with Eco RI, an enzyme which fails to recognize cleavage sites in this proviral strain. One mink cell clone was found to contain a single proviral DNA-containing fragment of 14 kb which was partially purified and subsequently cloned in a bacteriophage vector. The cloned DNA fragment contains the entire transforming provirus of GA-FeSV (6.7 kb) flanked by mink host cellular sequences. Restriction mapping and heteroduplexing were used to compare the GA- and ST-FeSV DNAs. GA-FeSV was found to contain all of the sequences represented in ST-FeSV as well as two additional regions of nonhomology which have no counterpart in ST-FeSV DNA. The additional segments account for the larger comparative size of the GA-FeSV provirus (1.7 kb).

The two nonhomology regions include (i) 1.2 kb of additional env-derived sequences 3' to v-fes, and (ii) a 0.5 kb sequence interposed between gag and the 5'end of the v-fes homology region. The latter sequence falls within the polyprotein coding region and explains why the the GA-FeSV gag-x product (P95) is larger than that of ST-FeSV (P85).

The presence of additional sequences within the polyprotein coding region of the GA-FeSV genome raised the possibility that the GA-FeSV product was partially specified by additional information derived from c-fes, FeLV, or both. A 0.4 kb Pst I fragment containing 200 base pairs from the 0.5 kb nonhomology region as well as 200 base pairs from v-fes was subcloned into pBR322. The failure of this plasmid DNA to hybridize to the FeLV genome suggested that at least some of the extra GA-FeSV sequences in the polyprotein coding region were derived from the cat onc gene. This was confirmed by hybridizing the plasmid with cat DNA which identified additional sequences upstream from previously mapped c-fes exons. The presence of a unique c-fes-derived segment in GA-FeSV shows that not all segments of the c-fes gene are required for transformation.

The intact 6.7 kb GA-FeSV provirus was recloned in pBR322. Subgenomic fragments representing different portions of the GA-FeSV genome were also subcloned, and several deletion and substitution mutants were prepared by a combination of chemical and recombinant DNA procedures. Transfection of different subcloned elements onto NIH/3T3 cells established that the left-hand LTR region and the contiguous polyprotein coding region were required for high efficiency focus-formation (2000-5000 ffu/pmole DNA). Sequences 3' to the v-fes coding region were not necessary for transformation. From these studies, it seems most likely that the transcriptional promotor, the 5' cap signal, the ribosomal binding site for mRNA, ATG codons (the gag aminoterminus), and the polyadenylation signal are contributed by FeLV-derived sequences, resulting in the high levels of transcription and translation of mRNAs containing v-fes sequences in cells transformed by these viruses.

Cells transformed by GA-FeSV (by viral infection or after DNA transfection) synthesized a gag-x polyprotein (P95). Like the ST-FeSV coded product, P95 exhibited tyrosine-specific PK activity and was, itself, phosphorylated in tyrosine in vivo. Both phosphorylated and glycosylated forms of GA-FeSV could be detected in GA-FeSV-transformed mink, rat, and mouse cells. It is concluded that GA-FeSV has transduced similar but nonidentical segments of a cat cellular gene as those found in ST-FeSV and that both viruses encode antigenically and chemically related proteins with PK activities which are required for expression of the transformed phenotype.

M-FeSV: Labeled cDNA transcripts containing v-fes sequences were previously reported not to hybridize to M-FeSV viral RNA suggesting that the latter FeSV strain contains a different transforming gene (here designated "v-fms") than that of ST- and GA-FeSV. Using techniques analogous to those described for GA-FeSV, a 10 kb fragment containing the complete M-FeSV provirus was molecularly cloned. Restriction endonuclease analyses and heteroduplexing established that M-FeSV is one of the largest known sarcoma viruses described to date (about 8.5 kb). The order of genes in M-FeSV is 5'-gag-fms-env-3'; virtually the complete FeLV gag gene (p15, p12, p30, and part of p10) and all of the FeLV env gene are represented and flank fms sequences of 3.0 - 3.5 kb in length.

Unlike ST- and GA-FeSV, M-FeSV codes for several different polypeptides. The largest of these is approximately 170-180,000 daltons (P180) and appears to represent the complete coding capacity of gag and fms sequences. A P120 product was also found which lacked gag antigenic determinants and contained antigens specified only by fms sequences. Whether p120 is synthesized from a separate mRNA species than p180 or represents a cleavage product of the larger precursor remains unclear. Neither P180 nor P120 have been found to specify PK activity. M-FeSV also codes for a complete envelope glycoprotein precursor (gp85) which can be detected both in mink and rat cells transformed by the virus.

Comparative restriction endonuclease mapping, heteroduplexing analyses, and nucleic acid hybridization experiments have thus far failed to establish any cross-homology between v-fms and v-fes sequences. Taken together with data concerning the size and antigenicity of v-fms coded polypeptides, it appears that fms represents a second, distinct oncogene transduced by FeLV from normal cat cellular DNA.

Significance to Biomedical Research and the Program of the Institute:

Molecular cloning and genetic engineering now permit detailed structural comparisons and genetic analyses of RNA tumor viruses at the level of DNA. These approaches have facilitated the first formal demonstration that feline sarcoma viruses are natural genetic recombinants between a viral vector (the feline leukemia virus) and segments of at least two different cellular genes which are represented in the DNA of normal cats. Recombination places genes which are normally repressed under the control of viral regulatory elements; this allows the genes to be transcribed at high levels and leads to augmented expression of the transduced gene's product. In the case of one of these genes (v-fes), the "transforming protein" is now known to be a protein kinase with specificity for tyrosine residues. The realization that several different groups of avian and mammalian tumor viruses have transduced genes with similar functions provides the first demonstration that the mechanism of transformation for each of these viruses may be similar. It is now becoming apparent that each of these products has a counterpart in normal cells, suggesting that activation of "oncogenes" may in fact involve induction of enzymes whose participation in an interrelated metabolic pathway is crucial in the regulation of growth.
Of the dozen known oncogenes now known to be represented in the acutely transforming RNA tumor viruses, two such elements have been identified in the cat system. The ability to purify these genes and characterize them in detail provides the first opportunity to probe for homologous elements in human DNA and to isolate genes putatively involved in human neoplasia.

Proposed Course:

Cellular sequences related to v-fes and v-fms will be molecularly cloned from the DNA of normal cells. Molecular cloning of integrated FeSV proviruses has generated the necessary expertise and experience with host-vector systems sufficiently powerful to be useful in the isolation of single copy genes. Plasmids containing v-fes sequences are already available and have been characterized in detail; by contrast, analogous recombinant subclones containing fms sequences must be constructed before these can be effectively utilized as probes. A variety of recombinant plasmid and phage clones have been supplied to numerous laboratories both within and outside NIH; these have already been used to generate new clones of interest which will facilitate work in this and related areas.

Two avian viruses, the Fujinami and PRC II strains, have recently been demonstrated to contain onc elements homologous to v-fes. As predicted from nucleic acid hybridization experiments, both of these avian sarcoma viruses are now known to encode tyrosine-specific protein kinases which cross-react antigenically with the ST- and GA-FeSV coded polyprotein molecules. These data provide the first example of transduction of the same cellular gene from different species and suggest that the number of transducible oncogenes may, in fact, be relatively few. Clones of ST- and GA-FeSV have been provided to outside investigators interested in the homologous avian sarcoma viruses, and attempts will be made to clarify the extent of homology among the different cellular genes.

Further attempts will be made to more precisely characterize sequences within the polyprotein coding frame. The results of molecular cloning and nucleotide sequencing analyses suggest that only portions of the v-fes sequence may be critical for PK activity, while other portions of the coding sequence may be modifiable. Moreover, it is clear that two forms of the ST- and GA-FeSV polyprotein which differ in their patterns of glycosylation and phosphorylation are synthesized in vivo, only one of which may be responsible for transformation. Presumably the differences in these proteins are conferred by signal sequences localized to the gag leader region. These results raise the possibility of generating mutants which code for products whose location within cells is altered (topological mutants) or, alternatively, which synthesize products capable of competing with the wild-type transforming signal (dominant defectives). A series of different mutants of GA-FeSV is being prepared using techniques of site specific mutagenesis. These will be tested for transforming capability as well as for their ability to encode nontransforming protein variants.

The LTR regions of the FeSV strains are also of interest since they contain the transcriptional promotor elements. Since the promotor sequences are recognized by cellular polymerases, we are testing the possibility that "promotor strength" is species-specific. The failure of FeSV DNA to transform mouse cells at an efficiency attainable with certain rodent recombinants suggests that substitution of mouse LTR sequences might augment the apparent transforming efficiency of cloned FeSV DNA on NIH/3T3 cells. Similarly, the FeSV LTR element may facilitate improved transcription of other onc elements in carnivore cells.

Rapid progress in defining the role of the transforming gene products and their potential metabolic interrelationships necessitate the purification of the proteins and their eventual biochemical characterization. In order to generate a better detection system, we plan to prepare monoclonal antibodies directed toward the FeSV gene products. Because tumor-bearing rats are known to mount an immune response to FeSV-transformed isogeneic rat cells and synthesize high titer antibodies to the FeSV gene products, we plan to use a rat cell system (rat splenocytes x rat myeloma cells) to generate somatic cell hybrid clones. Antibody producing clones will be screened using a modified system for immunoprecipitation of metabolically labeled substrate followed by electrophoretic analysis of the products. Such a system has already been employed in Dr. Scolnick's laboratory (LTVG) to prepare monoclonal antibodies to Harvey sarcoma virus p21 protein.

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CARCINOGENESIS INTRAMURAL PROGRAM

SUMMARY REPORT

LABORATORY OF BIOLOGY

October 1, 1980 through September 30, 1981

The Laboratory of Biology plans and conducts *in vitro* and *in vivo* investigations of the modulation of transformation that results in cancer. Modulation is an important approach to understanding the biological and molecular mechanism(s) that lead to cancer. The primary objective is to determine the crucial molecular changes that occur in cells, treated with chemical or physical agents, as they transform from the normal to the neoplastic state. Coordinated biochemical and biological studies are used (1) to characterize the cellular alterations associated with carcinogenesis; (2) to evaluate the relationships between DNA metabolism and carcinogenesis; (3) to determine the effect of physiological host mediating factors on carcinogenesis; and (4) to develop new *in vitro* cellular transformation systems which are pertinent for the study of the molecular mechanisms of carcinogenesis.

In vitro cell culture models have permitted the study of factors which influence mammalian cell transformation, a primary step in neoplasia, as well as the study of environmental agents that are potential carcinogens. An understanding of the mechanisms of carcinogenesis should make possible the development of information critical to planning interventive measures in the carcinogenesis process. Reversing or inhibiting the genesis of cancer is the crossover area of cancer prevention and therapy.

While the molecular basis for carcinogenesis is still largely unknown, DNA repair appears to play an important role. This is inferred from the fact that patients with rare genetic diseases such as xeroderma pigmentosium and ataxia telangectasia are both cancer prone and also have a cellular DNA repair defect. How DNA repair is involved in carcinogenesis is an enigma. Therefore, DNA metabolism in relation to transformation is being studied: a) to demonstrate that DNA damage is the crucial step in the initiation of carcinogenesis; b) to elucidate the specific DNA alterations involved in carcinogenesis; and c) to understand the role of various DNA repair mechanisms in carcinogenesis, cocarcinogenesis, and anticarcinogenesis.

A dilemma has existed in carcinogenesis in the sense that although a number of carcinogens are known to cause cancer in humans and experimental animals, demonstrating the ability to transform human cells or obtaining reproducible models for transformation by chemical carcinogens *in vitro* has been difficult. It is now possible to transform a human embryonic lung fibroblast cell strain, MRC-5, by a variety of chemical carcinogens, to obtain quantitative responses using growth in agar as an end point, and to demonstrate that cells derived from colonies that grew in agar produced tumors. The success obtained with the variety of carcinogens is attributed to the effectiveness to the blocking of cells in the G_1 period. A number of chemical agents, primarily of a hormone nature, are able to sensitize the cells to transformation when added during early S period. These observations add further evidence that DNA metabolism is a central point in the carcinogenesis process. The utilization of a well-defined cell strain, such as the MRC-5, has potential value as a new model for the study of carcinogenesis of human cells, as well as for investigating the relationship of transformation and the aging phenomenon.

The first UV action spectra for both transformation and pyrimidine dimer production in the same mammalian cells has been determined. We have measured, concurrently, the action spectra for UV-induced transformation, lethality, and pyrimidine dimer (the major UV-induced photoproduct in DNA) induction in hamster embryo cells (HEC). The relative sensitivities per quantum for transformation, pyrimidine dimer production, and lethality are essentially the same for the wavelengths tested. The most effective wavelengths were 265-270 nm. Since the action spectra for these three effects are essentially the same, the results add strong support to the concept that DNA is the target of UV-induced carcinogenesis.

The transformation frequency associated with a chemical carcinogen can be increased by sequential use of other chemicals or physical agents. Since no correlation exists between changes in the rate of excision or post-replication repair and enhancement of transformation, other repair mechanisms must be responsible for cell survival, as well as enhanced transformation. After cells are irradiated with UV, two modes of DNA replication are observed. During the early mode that operates for the first 3-4 hr post-UV, nascent DNA strands were smaller than those in nonirradiated cells. During the late mode, the nascent strands were of normal size. Incubating the cells with caffeine post UV-irradiation (10 J/m^2) inhibited the conversion of the early mode to the late mode. The change from early mode to late mode replication occurs at about 4 hr post-UV and with the time interval for caffeine's greatest effect on transformation. The observed caffeine-induced changes in the post-UV DNA replication can account for the potentiation of UV-induced lethality by caffeine and may be partially responsible for the enhancement of transformation.

Genetic damage caused by most carcinogens is visible in metaphase chromosomes as structural aberrations or sister chromatid exchanges (SCE). They are compatible with cell survival. In general, chemical and physical agents that are good inducers of SCE are good transforming agents. The correlation between induced SCE and specific locus mutations for several agents that differ in their mode of interaction with DNA has been shown. The question arises as to the relevances of SCE in the process of in vitro transformation. When sister cultures of HEC were used for SCE and transformation assays with a variety of carcinogens, all the chemical carcinogens produced a dose-dependent increase in SCE and transformation frequency, demonstrating the sensitivity of both assays to carcinogens. The ratios of induced SCE relative to transformation frequency, however, varied with the carcinogen. The positive linear correlation between carcinogen-induced SCE and transformation suggests a relationship between the two cellular responses. The number of SCE per cell induced by carcinogens far exceeds the transformation per cell. In the absence of chemical or physical treatment, all cells exhibit some SCE, but not morphological transformation. Therefore, a special class of induced SCE involving unequal exchange of DNA may represent DNA lesions relevant to the process of cell transformation.

In recent years, the phenomenon of initiation-promotion has been shown to occur in vitro. An important aspect of our in vitro transformation studies is to elucidate the role of chromosomal changes in carcinogenesis. Therefore, a coordinated transformation and cytogenetic study was designed to determine the relationship of the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), to chromosomal changes during the process of transformation. Whereas TPA was ineffective in influencing transformation frequency by itself, it did enhance transformation obtained with N-methyl-N'nitro-N-nitrosoguanidine (MNNG) and a series of hydrocarbons known to be weak or noncarcinogens. The enhanced transformation of frequency indicates that the number of cells initiated by a low dose of carcinogen is greater than those cells transformed by a carcinogen alone. TPA alone was ineffective in inducing SCE or in altering SCE frequency in MNNG cells that were growing logrithmically or had been treated with TPA and released from confluency. TPA post MNNG did not alter the percent of cells with aberrations or the total number of aberrations per 100 metaphases, and did not induce SCE in V79-4 Chinese hamster cells or alter the metaphases. TPA failed to induce SCE in V79-4 Chinese hamster cells or alter SCE frequency associated with MNNG. A lack of effect by TPA is consistent with the evidence that TPA does not covalently bind with DNA.

Post-carcinogen treatment, but not pretreatment, with varying concentrations of antipain also inhibits transformation; the transformation frequency, however, is unaffected by MNNG, antipain, or the combination of the two; no synergistic lethality occurs as reflected in the cloning efficiency of the various groups. MNNG treatment causes a variety of chromosomal aberrations, primarily of the chromatid type, and consists of gaps, breaks, and exchanges. No specificity in terms of chromosome involvement or chromosome segments was noted. With the Syrian hamster cells, larger chromosomes appeared more often to be the target of damage. Because chromosomal abnormalities are often associated with neoplasia, a relationship has been assumed between changes in chromatid function and cancer. On the basis of the average number of SCE per metaphase or chromosome, antipain did not alter the 5-bromodeoxyuridine induced rate of untreated controls or the frequency of MNNG induced rate when added 10 min post- or 24 hr pre-MNNG. However, our results with HEC that undergo transformation demonstrate an increased frequency of chromosomal aberrations (per metaphase) when treated with MNNG followed by antipain, the same procedure used for the transformation studies. The increase in the number of aberrations per metaphase with MNNG and antipain compared to MNNG alone was statistically significant at 10, 26, and 40 hr after treatment. Although the mode of action of antipain is unknown, it is unlikely that the mechanism of gene conversion leading to transformation involves a protolytic sensitive step that controls the formation of chromatid exchanges. The reduction in transformation frequency may simply reflect cell death due to the increase in chromosome aberrations and thus may be responsible for eliminating a subpopulation that was destined to be transformed.

It has been intellectually more satisfying and consistent with the irreversibility of the initiating event to presume that a mutation-like process occurs very rapidly and that the long latent period of carcinogenesis involves the promoting phase. The time dependence for enhanced transformation with X-ray and alkylating agents followed by a potent carcinogen or UV suggested that mechanisms other than point mutations are responsible for survival of hamster cells and for the radiation type enhanced transformation. Since the damage persists and cells survive with that damage, DNA alteration is probably responsible for the transformation. What is unresolved is whether DNA alteration leads to mutation(s) that are involved in the conversion of nonmalignant cells to malignancy. In the hamster in vitro cell model of carcinogenesis in which the changes can be observed rapidly and in a quantitative fashion, studies in the immunological prevention or inhibition of carcinogenesis have been extended to evaluate the role of lymphoid cells and lymphokines in carcinogenesis. Previous studies indicating that lymphotoxin, a cytotoxic lymphokine, can irreversibly inhibit both chemical or ultraviolet carcinogenesis have been extended to macrophages and lymphocytes from normal hamsters. Macrophages such as lymphotoxin are able to inhibit carcinogen-induced transformation in proportion to their number in the assay. Lymphocytes, however, induce a biphasic response reminiscent of the stimulation-inhibition type pattern observed in the intact animal during carcinogenesis. The addition of lymphocytes causes a diminuition in transformation frequency as the number of lymphocytes increases at a ratio of approximately 100 to 1, by an increase in the frequency of transformation and a subsequent decrease again at the lymphocyte target cell ratio of 1000 to 1. This inhibition or prevention of transformation is abolished and the inhibition becomes linear if the lymphocytes are stimulated by either an antigen or a mitogen. Whether the phasic response is due to a subpopulation of lymphoid cells or to lymphokines or a combination of the two, or whether this mechanism might also operate in vivo is unknown. If the latter should be the case, the abolishment of the phasic response by mitogen or antigen stimulation suggests that a state of hyperimmunity could be associated with a decreased incidence in tumor formation. Thus, studies also indicate that whereas the lymphotoxin induced inhibition of carcinogenesis is irreversible, the inhibition of tumor cell growth in culture is reversible. The latter also requires much greater concentrations of lymphotoxin for inhibition than do the earlier stages of carcinogenesis.

Biochemical analysis of cells following exposure to lymphotoxin reveals an increase in high molecular weight cell membrane glycoprotein synthesis in nontumorigenic Syrian hamster cells and an inhibition in the synthesis of the same glycoproteins in proportion to lymphotoxin concentration in malignantly transformed cells. These qualitative and quantitative lymphotoxin-induced alterations in membrane glycoproteins correlate with the differential cytotoxicity of lymphotoxin for malignant cells compared with nontumorigenic cells. The stimulation of glycoprotein synthesis by lymphotoxin in nontumorigenic secondary passaged cells demonstrates that lymphotoxin can alter the physiological state of nontumorigenic cells and may indicate part of the molecular mechanism(s) whereby lymphotoxin can prevent carcinogenesis.

Adrenocorticosteroids are another physiological means whereby the host can alter carcinogenesis. These steroids have been shown to inhibit the development of both complete and phorbol ester promoted polycyclic hydrocarbon-induced experimental tumors. Investigations this past year have shown that physiological concentrations of corticosteroids can inhibit ultraviolet-induced hamster cell transformation without inhibiting cell proliferation. There is a strong temporal relationship between the addition of adrenocorticosteroids and carcinogens. Maximal inhibition of carcinogenesis is obtained when the cells are exposed to the steroids prior to the addition of carcinogen, but little effect upon carcinogenesis when the steriod is added several days after carcinogen treatment. This temporal relationship is potentially very important when one considers the diurnal variation in circulating steroid levels in the intact animal in relationship to the time of carcinogen exposure.

The demonstration that the in vitro transformation phenomena can be modulated has made possible the study of cocarcinogenesis and anticarcinogenesis at the target cell level. A number of biological, immunological and cytogenetic probes are providing valuable information for understanding the mechanism for the induction of cancer and making possible the inhibition of the carcinogenesis process. The studies of the Laboratory of Biology suggest that there are many types of initiation in carcinogenesis and that the physiological state of normal cells is an important factor in determining whether the cells will be sensitive to a transforming agent that leads to neoplasia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04629-16 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Mechanism of Cell Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.A. DiPaolo	Chief, Laboratory of Biology	LB NCI
OTHER:	J. Doniger	Expert Scientist	LB NCI
	C. H. Evans	Chief, Tumor Biology Section	LB NCI
	J. W. Greiner	Staff Fellow	LB NCI
	M. L. Larramendy	Visiting Fellow	LB NCI
	N. C. Popescu	Visiting Scientist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biology, Carcinogenesis Intramural Program

SECTION
Somatic Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The conditions responsible for modulation of in vitro transformation are being investigated, with specific emphasis of the use of UV, X-rays, combinations of carcinogens of different classes, the combination of in vivo - in vitro systems, and the effect of repair mechanisms on toxicity and transformation. The relevance of chromosome damage as a result of initial carcinogen insult, as well as to which cytogenetic alterations are responsible for or associated with malignancy is being determined using cells from a variety of species including human. Studies on the mechanisms of early events of carcinogen cell interaction and investigations of different proximate carcinogens and metabolic inhibitors are emphasized. The requirements necessary to obtain reproducible neoplastic transformation of human fetal fibroblasts and adult epithelial cells by chemical carcinogens are being determined. The testing for the tumorigenicity of cells exposed to different agents for different time intervals will continue.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objective of this project is to establish conditions and methods for in vitro quantitative study of chemical transformation. The immediate areas of study are:

1. Development of rapid assay systems for chemical carcinogens suitable for studying the modulation of transformation.

2. Define the conditions required for the transformation of human fetal cells.

3. Determine ways to increase the susceptibility of primary cell lines or cell strains to chemical transformation.

4. Development of assays to determine whether in vitro transformation is accompanied by the appearance of tumor specific antigens.

5. Determine the role of nononcogenic and oncogenic viruses in chemical transformation.

6. Determination of the ultrastructural changes of cells during the course of in vitro chemical transformation so that they may be contrasted with normal cells and with information pertaining to viral transformation.

7. Determine the role of DNA repair, e.g. rate, excision and post-replication in enhancing neoplastic transformation with radiation and alkylating compounds.

8. The transformation of epithelial-like cells which result in the formation of carcinomas when the cells are transplanted into animals.

9. Analysis of variations in populations of somatic cells and their correlation with genetic changes.

Methods Employed: All procedures performed are with the view of quantitating phenomena in vitro. Such procedures are required to determine whether or not the transformation observed is due to the direct or indirect effect of the carcinogen and in order to study the early events associated with in vitro transformation. Cells are derived from freshly isolated cells from animals and humans that as controls have many of the attributes of "normal" cells and from cell lines which are known to exhibit some of the properties associated with nontransformed cells. Discrete cells are grown in complete medium in the presence or absence of irradiated rat or hamster cells. The cells may be derived from whole embryos or may be from specific organs. The cells are exposed to chemical carcinogen transplacentally or prior to or subsequent to seeding the cells in Petri dishes.

Approximately one week subsequent to treatment, the cells are examined under phase or under stained conditions for number of transformed colonies, toxicity, and spectrum of morphology of both the normal and transformed colonies. The frequency of transformation is expressed in a number of different ways. These take into consideration the observed rate of transformation on a per-cell basis or on a number of colonies obtained.

Major Findings: The addition of 12-O-tetradecanoylphorbol-13-acetate (TPA) to nontransformed colonies of X-irradiated (200 R) Syrian hamster embryo cells (HEC) results in the morphologic transformation of 5-10% of the surviving colonies. We determined 1) whether TPA-promoted transformation is permanent and 2) whether X-ray or ultraviolet light (UV)-induced initiation and/or TPA-promoted transformation cause changes in the cell surface that make the cells sensitive to lymphotoxin (LT), a natural biological inhibitor of transformation. Primary HEC were seeded (300 cells/60 mm dish) with X-irradiated feeder cells for cloning and 1 day later the cells were either X-(200 R) or UV- (4.5 J/m^2) irradiated. TPA and/or LT were added post-irradiation. Seven days after seeding, the cells were stained and scored for transformation and cloning efficiency. When TPA was added to the cultures 2, 3, or 4 days post-X-ray, the percent of transformed colonies was essentially the same. In the 4 day post-X-ray group, the cells were exposed to TPA for only 2 days. When TPA was added 2 days post-X-ray and removed 2 days later, the transformation frequency was 55 to 80% less than when TPA was not removed. Therefore, the presence of TPA is necessary to maintain the transformed phenotype of the majority of the X-ray initiated cells. The addition of LT to HEC between X-ray and TPA reduced TPA-promoted transformation by 80%. UV-induced transformation was enhanced 15-20 fold when TPA was added 2 days post-UV. Much (70%) of this increase was prevented by LT addition 2 days post-TPA. These studies show that initiated HEC are phenotypically transformed in the presence of TPA, but are not necessarily committed to be transformed and that both initiated and promoted HEC are sensitive to LT, suggesting that the physiological state of normal cells is important for transformation.

Serially subpassed human cells grow in vitro as randomly proliferating monolayer cultures with subpopulations capable of different rates of scheduled and/or unscheduled DNA repair synthesis. Cells derived from primary foreskin and the MRC-5 cell strain are transformed by a variety of carcinogens. A linear relationship between dose of carcinogen and the acquisition of anchorage independent growth occurs. Furthermore, colony growth in agar is indicative of an inductive phenomenon and implies dose dependency. The expression of the transformed phenotype after a carcinogenic insult requires preferential cell multiplication. The failure of cell proliferation and fixation of the initial transformation event result in the chemically treated cells becoming part of the cell population belonging to a permanent resting phase of the cell cycle. In this way, prevention or suppression of cell transformation would occur. Chemical carcinogen-induced transformation of human cells occurs more efficiently in low passage populations that are first blocked in G_1, released from the block, then treated in S period. Arginine- and glutamine-deficient medium was required to effectively block the cells in the G_1 period. Estradiol, insulin, anthralin or phorbol myristate acetate sensitized the cell population to carcinogen treatment when added 10 hr before the carcinogen in early S period. Presensitized cells kept blocked in G_1 period for 48 hr or longer, released and treated in S period with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or aflatoxin B1 were not transformed; nor did transformation occur in presensitized cell populations treated in G_2 (4.5 hr), M (1.5 hr) or G_1 (8.2 hr). Cells derived from carcinogen-treated presensitized cells grew as colonies in soft agar and when cells derived from colonies isolated from the soft agar were injected subcutaneously into nude mice, tumors developed.

Asbestos and benzo(a)pyrene (BP) act synergistically in the transformation of Syrian hamster embryo cells. Epidemiological evidence has clearly demonstrated an association between exposure to asbestos dust, a high incidence of carcinoma of the lung, and a high frequency of rare neoplasms, mesotheliomas of the pleura and peritoneum; in fact, the incidence of pulmonary cancers among asbestos workers who are cigarette smokers is 92 times that of the general population. Four varieties of asbestos fibers, crocidolite, anthophyllite, amosite, and chrysotile, induced a low rate of morphologic transformation in Syrian hamster cells. Of the four tested, chrysotile was the most lethal as reflected by colony survival. When cells were exposed to 1 μg of BP per 1 ml medium and two different concentrations of the asbestos fibers, an enhancement of transformation occurred with BP. The enhancement was dose responsive with all fiber species except for amosite which was dose independent. The synergism of transformation obtained with the combinations of asbestos fibers and BP did not occur with UV irradiation. The addition of crocidolite immediately after UV failed to increase the number of transformation per dish or the transformation frequency even though cell lethality was potentiated. The lethality by UV alone was consistent with that previously reported. The synergistic activity of BP and asbestos suggests that asbestos facilitates the transport of BP to the cell site(s) critical for transformation. These results provide a basis for investigating the carcinogenic and cocarcinogenic potential of asbestos fibers in mammalian cells.

Because cyclic nucleotides may regulate cell multiplication, cell morphology and synthesis of some macromolecules, it is important to determine the role of these compounds in neoplastic and malignant growth. Cyclic adenosine 3':5'-monophosphate (cAMP) and cyclic guanosine 3':5'-monophosphate (cGMP), or their dibutyryl and monobrominated derivatives, increase or decrease morphologic transformation of HEC exposed to MNNG. Concentrations of 5 mM or larger for a minimum 24 hr of cAMP, cGMP and their analogs were necessary to reduce the colony forming ability of nontransformed HEC, but cGMP and its derivatives caused less toxicity than cAMP or its congeners. cAMP and analogs reduced the transformation frequency, irrespective of concentration and when administerd relative to MNNG alone. The most inhibitory effects were obtained with Bt_2cAMP followed by 8BrcAMP and cAMP. At equimolecular doses the dibutyryl and brominated analogs of cGMP, but not unsubstituted cGMP, enhanced transformation when administered prior to MNNG but reduced the transformation frequency when added after carcinogen. The enhancing and inhibitory effects on transformation frequencies were dose- and time-dependent and occurred in the order Bt_2cGMP> 8BrcGMP>>cGMP. Butyric acid (BA), a metabolic derivative of Bt_2cAMP, neither diminished nor increased MNNG-induced transformation frequency. Thus, the modulating action of at least Bt_2cAMP and Bt_2cGMP on morphologic transformation induced by MNNG in HEC is related to the cyclic nucleotide part of these molecules. The effects of Bt_2cAMP and BtcAMP were similar when given after MNNG but different when administered prior the carcinogen. Since BA is formed in both cases from these dibutyryl analogs, other mechanisms have a higher probability of being involved. Variations in decomposition rate of the carcinogen and/or alterations in the DNA repair processes elicited by MNNG in HEC are probably important in modulating transformation of HEC by cyclic nucleotides.

Coordinated studies of DNA repair and in vitro transformation of HEC have been carried out to determine if the ability of caffeine to affect DNA replication in UV-irradiated HEC is related to caffeine's ability to enhance transformation induced by UV. Transformation is enhanced 2-5 fold when caffeine is added to HEC after UV. Maximum enhancement occurs when the interval between UV and caffeine is 4 hours. Caffeine affects the 2 modes of DNA replication observed in UV-irradiated HEC. During the early mode (from 0-4 hr post-UV), nascent daughter DNA strands are smaller than in nonirradiated cells. Caffeine inhibits the elongation of these small nascent strands to the size of parental DNA. During the late mode (after 4 hr post-UV), the size of nascent strands is the same as in nonirradiated cells. Caffeine inhibits the conversion of the early mode to the late mode, and when added during the late mode, caffeine causes reversal to the early mode. Therefore, caffeine perpetuates the small nascent strands induced by UV. The observed caffeine effects on the size of nascent DNA strands account for the potentiation of UV-induced lethality and may be important in transformation.

Action spectra were determined for neoplastic transformation, production of pyrimidine dimers, and lethality in HEC. Of wavelengths between 240 and 313 nm, the most effective were 265 and 270. The relative sensitivities per quantum for transformation, pyrimidine dimer production, and lethality were essentially the same at each of the wavelengths tested. This first action spectrum for transformation, which is relevant to carcinogenesis, is similar to spectra obtained previously by measuring other cellular responses in either microbial or mammalian systems. Because the action spectra for cytotoxicity and transformation are the same as the spectrum for dimer production, DNA is suggested as the target for all these processes.

Bisulfite is a compound that warrants extensive study for its effects at the cellular level because it is a food and pharmaceutical additive and it is a ubiquitous pollutant in the form of SO_2. It was recently reported that bisulfite, at neutral pH, does not act as a mutagen in either bacterial or mammalian systems but does enhance UV-induced mutation in both model systems. Therefore, bisulphite was tested in the HEC transformation system to determine if it is cocarcinogenic with UV. The results indicated that while there is no synergism for transformation with bisulphite plus UV, bisulfite alone is an effective transforming agent. The effect of bisulfite on DNA metabolism was also investigated. Bisulfite does not induce any excision or post-replication repair responses and does not effect UV-induced repair. However, it does inhibit overall DNA replication apparently by inhibiting a fraction of the replicons from operating. The results suggest that bisulfite induces transformation by an epigenetic rather than genetic mechanism.

In a collaborative study of structure-function relationships, we have measured transformation and lethality induced in HEC by a series of six vinyl chloride derivatives. When the results are compared to the chemical stability of compounds, the most stable were, in general, the most effective. However, two of the compounds that have the same stability differ in their ability to induce transformation by one order of magnitude. These results are compared to both _in vivo_ carcinogenesis, as well as direct bacterial mutagenesis. In general, _in vitro_ the transformation results correlate with the _in vivo_ results.

Several inorganic metal salts have been tested in the quantitative HEC system or by a host-mediated in vivo-in vitro assay for their ability to cause morphologic transformation. Whereas nickel, beryllium, cadmium, chromium and arsenic salts produced transformation of HEC, salts of iron, titanium, tungstate, zinc, aluminum and nickel sulfide amorphous did not. The transforming metals also enhanced transformation associated with Simian adenovirus-7. Epidemiological evidence exists for the carcinogenicity of salts of arsenic, nickel and beryllium. Although the mechanisms of action of metals are unknown, they either cause DNA breaks or decrease the fidelity of DNA synthesis. Either mechanism could be involved in transformation. Chromosomal aberrations and sister chromatid exchange (SCE) induction were determined with human and hamster cell strains. Arsenic, nickel and beryllium salts at concentrations that exist in the environment and that are effective in causing transformation of HEC-induced SCE and chromosome aberrations in human and hamster cells, whereas sodium tungstate was negative. Human and hamster cell strains exhibited an equal sensitivity to the metal-induced damage. The SCE and chromosome aberration induction by metal salts correlates with in vitro morphological transformation. The significance of the two cytogenetic responses to carcinogenesis is different as chromosome aberrations are primarily associated with cell lethality, whereas SCE are compatible with cell survival and may be more relevant to carcinogenesis. The effect caused by metals in human cells suggest that they are potentially hazardous in human. The use of nonhuman cells with the same sensitivity shows that their use can provide relevant information to human sensitivity.

We demonstrated that the promotional aspect of transformation can be studied. With the quantitative HEC model as the tumor promoter TPA enhanced the morphologic transformation induced by low transforming concentration of MNNG. This occurs without potentiation of cell lethality or changes in SCE frequency. Several conditions which might influence SCE induction by TPA have been investigated using HEC cell strain and a Chinese hamster V79 cell line. Heat-inactivated serum was used since there is a possibility that a serum component may interfere with TPA activity in vitro. Since 5-bromodeoxyuridine (BUDR) is required for SCE analysis and its concentration may control the level of BUDR incorporation into DNA and in turn affect the cell response to TPA, several concentrations of BUDR were used. SCE which represents DNA interchange between sister chromatids are ordinarily visualized after two rounds of cell replication in the presence of BUDR, thus at the second division post-TPA treatment. By labeling the cells with BUDR for only one replication cycle prior to TPA exposure, it was possible to measure SCE frequency at the first mitosis post-TPA treatment. Results were obtained with MNNG, an alkylating carcinogen, UV and X-irradiation as the inducing agents. There is a possibility that the effect of TPA on SCE frequency may depend on the type of carcinogen-induced DNA lesion, as well as the time of its addition relative to the inducing agent. UV and X-irradiation are physical carcinogens that differ in their mode of interaction with the DNA and in their ability to cause SCE; UV irradiation is a potent inducer of SCE, whereas X-irradiation, although a potent clastogenic agent, is a poor inducer of SCE. A series of experiments on HEC or V79 Chinese hamster cell line have demonstrated that the enhancement of transformation induced by physical or chemical carcinogens is not due to an increase mitotic recombination activity as evidenced by SCE formation. TPA was ineffective, independent of the cell used, culture conditions, level of BUDR incorporation into DNA, or time interval between carcinogenic insult and TPA treatment.

The treatment used for remissions of psoriasis and mycosis fungoides involves a clinical photochemotherapy regime designated as PUVA and consists of a combination of 8-methoxypsoralen (8MOP) plus high-intensity long wavelength ultraviolet radiation (UV-A). This combination is potentially deleterious to humans. The transforming capacity of the combination 8MOP and UV-A correlates with DNA damage as documented by SCE induction. Only the combined 8MOP (0.01 αg/ml) plus UV-A (7.5 J/m^2) produced a significant increase in SCE frequency per chromosome in normal human lymphocyte cultures and in a lymphoblastoid cell line. Increasing concentration of 8MOP and UV-A exposure inhibited cell progression without increasing SCE incidence. The toxicity caused by 8MOP and UV-A is probably due to the effect on DNA synthesis as 8-MOP causes interstrand DNA crosslinks. SCE formation, however, does not correlate with the formation of DNA - DNA crosslinks. Therefore, the increased number of 8MOP-induced DNA cross links induces toxicity without the formation of additional SCE.

The relationship between SCE and transformation induction by a variety of carcinogens was studied using HEC. All chemical carcinogens tested produced a dose response increase in SCE and transformation frequency demonstrating the sensitivity of both assays to carcinogens. The ratio of induced SCE relative to transformation, however, varied with the carcinogen. In all cases the number of SCE per cell far exceeded the transformation per cell. In the absence of chemical carcinogen treatment, all cells exhibit some SCE but not morphologic transformation. It is postulated that a special class of induced SCE involving unequal exchange of DNA may represent DNA lesions relevant to the process of transformation. Although SCE and transformation induction may be related cellular responses, the efficiency of SCE induction relative to transformation depends on several factors: type of carcinogen-induced lesion, the ability of the target cell to repair the damage, and tissue type or species used. The latter factor becomes more important when _in vitro_ data are extrapolated to _in vivo_ condition for assessing human hazard. SCE base line was similar for all species examined, including humans independent of these diploid chromosome number. Thus, the normal SCE frequency correlates with the DNA content and not the chromosome number. Furthermore, normal cells and cells derived from tumors or permanent cell lines were also similar. However, when normal cells and permanent cell lines were subjected to MNNG treatment, the SCE frequency of permanent cell lines was significantly higher than that of normal cells. This may reflect a decreased repair capacity associated with the acquisition of uncontrolled growth. MNNG, however, induced a lower incidence of SCE in fibroblasts derived from fresh human foreskin specimens than in normal Syrian or Chinese hamster embryo cells. The response of primary human cells may reflect a more efficient repair of DNA alkylation and contribute to the difference in the response of human versus Syrian hamster cells during the process of morphologic transformation by chemical carcinogens.

Although carcinogens usually increase the SCE frequency, the tumor promoter, TPA, enhanced morphological transformation frequency induced by low concentrations of MNNG without potentiation of cell lethality or changes on SCE frequency. There is a possibility that a number of factors might influence SCE response to TPA: the nature of carcinogen-induced DNA lesion preceding TPA treatment, concentration and incorporation of BUDR into DNA, number of cell divisions post-TPA exposure or type of serum used for cell culturing. Since a serum component may interfere with TPA activity *in vitro*, HEC and a V79 Chinese hamster cell line were grown in medium supplemented with heat-inactivated serum. In a series of experiments, TPA did not produce an increase in SCE incidence independent of the type of serum, BrdUrd concentration and its level of incorporation into DNA or the number of cell divisions post TPA treatment. Furthermore TPA was ineffective in further increasing SCE frequency induced by UV and X-irradiation, two physical carcinogens that differ in their mode of interaction with DNA and efficiency of SCE induction. Therefore, enhancement of transformation associated with TPA is not due to an increase in mitotic recombination activity.

Significance to Biomedical Research and the Program of the Institute: The prevention of cancer in humans depends to a large extent on understanding the process that is responsible for the development of transformation and on removing potentially harmful environmental agents. The determination of factors responsible for transformation serves as basis for the study of mechanism(s) involved in transformation. In this way, it will be possible to intervene with or prevent the development of cancer. The cellular approach with human and animal cells will make it possible to study how to block, reverse, or eliminate transformed cells. In the process, biologically valid techniques for identifying potential carcinogens relevant to humans will also be developed.

Proposed Course: The overall approach to problems in carcinogenesis will be to continue to investigate the modulation of transformation of mammalian cells, vital aspects in the etiology and prevention of cancer. Whereas cell biology was emphasized in the past, molecular biological approaches are increasingly important in the elucidation of the objectives of the laboratory. Our specific objectives are (1) to define the role of chemical, physical and biological agents pertinent to the process of carcinogenesis; (2) to characterize the cellular alterations associated with carcinogenesis; (3) to evaluate the relationships between DNA (chromosome) and carcinogenesis; and (4) to probe the somatic mutation aspects of experimental carcinogenesis.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04673-10 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Immunobiology of Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. H. Evans	Chief, Tumor Biology Section	LB NCI
OTHER:	R. P. McCabe	Senior Staff Fellow	LB NCI
	J. A. DiPaolo	Chief, Laboratory of Biology	LB NCI
	J. H. Ransom	Staff Fellow	LB NCI
	J. P. Fuhrer	Expert Scientist	LB NCI
	J. A. Heinbaugh	Biologist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biology, Carcinogenesis Intramural Program

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3.0	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Natural immunity can inhibit the development of a tumor cell during carcinogenesis as evidenced by cytostatic and cytocidal assays during chemical carcinogen induction of neoplastic transformation in cultured mammalian cells. Natural cytotoxic activity of normal unstimulated nonimmune macrophages and lymphocyte populations for tumor cells is quantifiable by lymphoid effector cell-mediated inhibition of tumor cell colony formation and release of radionuclides. Lymphotoxin, one of the lymphokines, soluble mediators of immunoregulatory function, also exhibits cytotoxicity for tumor cells not observed with nontumorigenic cells. In in vitro models of carcinogenesis, such as neoplastic transformation of guinea pig cells, where distinct discrete stages in carcinogenesis can be readily identified and studied, susceptibility to natural immunocytotoxicity develops concomitantly or in close association with neoplastic transformation. Neoplastic cells, but not their nontumorigenic counterparts, also exhibit delayed hypersensitivity skin reactions in normal nonimmune syngeneic animals. This natural skin reactivity, like natural immunocytotoxicity, is independent of cell surface tumor-specific transplantation rejection antigens yet is individually distinctive for each tumor cell line.

Project Description

Objectives: The primary objective of this project is to elucidate at the target cell and host levels the relationships between cell surface alterations accompanying the development of neoplasia in the phenomenon of carcinogenesis. Specific objectives include: (1) identification of somatic cell alterations during carcinogenesis using in vitro model systems to allow study of membrane and other phenotypic changes at specific steps or stages in carcinogenesis, and (2) investigation of host interactions with specific cell surface alterations during carcinogenesis in vitro and in vivo. Particular emphasis is placed on natural and induced cellular and humoral immunobiological interactions due to the frequent occurrence of neoantigens, reexpression of fetal antigens, and alterations in alloantigens on tumor cells.

Methods Employed: Guinea pig, human, mouse, rat, and Syrian hamster cells are utilized in this study. The quantitative in vitro chemical carcinogenesis system developed within the Laboratory of Biology employing Syrian hamster embryo cells (Project Z01-CP-04629, The Mechanism of Cell Transformation) forms the basic methodology for obtaining normal, preneoplastic and neoplastic cells for study. Freshly isolated cells are obtained from embryos, fetuses or adult animals, exposed in utero or in vitro to chemical or physical carcinogens. The cells are cultured and studied for somatic cell changes such as altered morphology, morphological transformation, growth in agar, tumorigenicity and interaction with components of the immune system. Immunobiological techniques including direct and indirect immunofluorescence, complement fixation, colony inhibition, radionuclide uptake and release assays, delayed hypersensitivity skin reactions, and tumor transplantation rejection are employed in analyzing cell membrane changes and in assessing host interactions to the changes.

Major Findings: 1. Carcinogenesis utilizing guinea pig cells proceeds in discrete stages that frequently are displayed for extended periods of time. Morphological alterations relative to controls develop shortly after carcinogen exposure and persist for months. After four or more months, morphologically altered cells proceed to the stage of morphological transformation characterized by random growth and piling up of cells. Neoplastic transformation, the ability of cells to produce tumors when inoculated into host animals, may be demonstrated at the time of morphological transformation or frequently may require additional months of subculture. This stepwise progression of the carcinogenic process occurs following treatment of cells obtained from fetuses in midterm and near-term gestation and with cells isolated from neonatal guinea pigs. A similar pattern occurs during in vitro carcinogenesis of a nontumorigenic morphologically oriented fibroblast-like guinea pig continuous cell line treated with carcinogen 14 months after introduction into culture. This indicates that the transformation frequency, latent period, and multiple stages characteristic of carcinogenesis can be independent of cell age or number of generations.

2. A number of somatic cell alterations, including the ability to form colonies in agar, secretion of plasminogen activator, intradermal skin reactivity in nonimmune syngeneic animals and susceptibility to the cytotoxic activity of lymphotoxin, nonimmune macrophages and nonimmune lymphocytes develop in close proximity or concomitantly with neoplastic transformation. These cell properties provide useful quantitative means to identify neoplastic transformation of cells

in culture and to investigate cellular characteristics essential to the development of the neoplastic state. They also indicate that natural immunity mediated by macrophages, lymphocytes, and the lymphokine, lymphotoxin, recognizes and may modify and even inhibit the development of a neoplastic cell. Lymphotoxin as well as macrophages and lymphocytes from nonimmune hamsters can inhibit chemical carcinogen, as well as ultraviolet irradiation-induced transformation of Syrian hamster cells. Lymphotoxin inhibition of hamster cell transformation is irreversible and proportional to the dose of lymphotoxin. Inhibition by lymphocytes, however, exhibits a phasic response at a lymphocyte to hamster target cell ratio around 200:1. The phasic inhibition is abolished when the lymphocytes are stimulated by antigen or mitogen. Macrophages exhibit a linear inhibition of transformation.

3. The neoplastic state is associated with a quantitative specific susceptibility to lymphotoxin. A wide range of lymphotoxin susceptibilities exist among different neoplastically transformed cells that are independent of the origin of the cells or the initiating chemical carcinogen. Neither chromosomal alterations nor detectable tumor-specific cell surface neoantigens are necessary for neoplastic transformation; furthermore, these factors are unrelated to lymphotoxin susceptibility. Colony formation in agar and lymphotoxin susceptibility can develop concomitantly with, or following the onset of, the stage of preneoplastic morphological transformation. They also can develop, although rarely, independently of one another and many cell generations and months prior to attaining the stage of neoplastic transformation. Natural intradermal skin reactivity, however, has yet to be observed until the stage of neoplastic transformation. This separation of lymphotoxin susceptibility and natural skin reactivity into two different stages of carcinogenesis is of interest as these are two potential points for immunobiologic modulation of in vivo carcinogenesis.

4. Guinea pig, human and mouse lymphotoxin exhibit different species specificities for neoplastic and nontumorigenic cells. Mouse lymphotoxin and mouse tumor cells appear to have little species specificity. Mouse lymphotoxin is more cytotoxic to tumor than to nontumorigenic cells regardless of the species origin of the cells and mouse tumor cells are susceptible to the cytostatic (colony inhibitory) and cytolytic (radionuclide release) activity of guinea pig, human and mouse lymphotoxins. Guinea pig and human lymphotoxin, however, possess distinctive species specificity that is generally cytostatic rather than cytolethal. For example, guinea pig lymphotoxin inhibits the growth of guinea pig, but stimulates the growth of human tumor cells in colony inhibition assays. In contrast, human lymphotoxin stimulates guinea pig and inhibits human tumor cells. Lymphotoxin susceptibility is qualitatively and quantitatively independent of detectable tumor-specific neoantigens. Each tumor or tumor cell line, however, exhibits its own characteristic susceptibility to natural immunocytotoxicity, as well as its own intradermal skin reactivity in nonimmune syngeneic animals. The reasons for these remain to be explained and may provide fundamental new understandings to basic immunology, tumor biology, and to host interactions modulating carcinogenesis.

Significance to Biochemical Research and the Program of the Institute: This project provides a means to study and understand how the individual, through the mechanisms of natural immunity, intervenes to suppress, inhibit or even enhance the growth of an incipient tumor cell during carcinogenesis. Natural cytotoxicity of macrophages, lymphocytes and lymphokines alone or in combination can now be studied at various stages of carcinogenesis to provide new insights into the immunobiology of cancer. As the host mechanisms and the target cell structures with which the immune effectors interact are delineated, it will be possible to investigate how the natural and induced immunity of the individual host may be augmented to suppress and even prevent the final aspects of carcinogenesis--the transition from the preneoplastic to the neoplastic state.

Proposed Course: Investigations will continue to define the mechanisms whereby neoplastically transformed rodent and human cells are preferentially susceptible to the cytotoxic activity of naturally immune host-derived cellular and humoral effectors. A major thrust will be a multidisciplinary investigative analysis of cell membranes relevant to differentiating tumor cells from normal cells. The approaches will include membrane structure changes and macromolecular differences in cellular behavior. This program will, in part, seek to explain how lymphotoxin preferentially binds to and inhibits tumor cells and why cell to cell contact is necessary in most natural cellular immunocytotoxicity. New technology in cell surface topography and cell separation using computerized cytometers and cell sorting cytofluorographs will enable resolution of the relationships between the cytostatic and cytocidal activities of macrophages, lymphocytes, and lymphokines to tumor cells. Definition of these relationships will clarify the species specificity of the effector mechanisms and our understanding of the role of *in vivo* natural immunity in the phenomenon of carcinogenesis.

Publications:

Sisskin, E. E., Weinstein, I. B., Evans, C. H., and DiPaolo, J. A.: Plasminogen activator synthesis accompanying chemical carcinogen-induced *in vitro* transformation of Syrian hamster and guinea pig fetal cells. *Int. J. Cancer* 26: 331-336, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05145-02 LB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell Surface in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard P. McCabe	Senior Staff Fellow	LB NCI
OTHER:	Charles H. Evans	Chief, Tumor Biology Section	LB NCI
	Joseph A. DiPaolo	Chief, Laboratory of Biology	LB NCI
	Nancee M. Cahill	Biologist	LB NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Biology, Carcinogenesis Intramural Program

SECTION
Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The guinea pig in vitro carcinogenesis model, in which preneoplastic sequential stages can precede neoplastic transformation, the acquisition of tumorigenicity, by two years is useful in establishing the relationship of various cell surface properties, both individually and as combined factors, to progressive tumor growth. In this model, stages and factors associated with carcinogenesis are studied separate from those properties found only in tumorigenic cells. Using this model, we have found that all tumorigenic cells produce plasminogen activating proteases in amounts which correlate with the (1) tumorigenic threshold inoculum, (2) ability to induce a delayed type skin reaction in non-immune guinea pigs and (3) susceptibility to guinea pig lymphotoxin. All tumorigenic cells produced reduced amounts of fibronectin and Forssman glycolipid but the amounts produced do not correlate with the tumorigenic ability of the cells. An inhibitor of plasmin activity was produced by the same cells producing the plasminogen activating enzymes suggesting that control of local in vivo proteolysis may include an antagonistic relationship between plasminogen-activating and plasmin-inhibiting activities.

Project Description

Objectives: In the guinea pig *in vitro* carcinogenesis system, several distinct and extended stages in the progression to neoplastic transformation can be identified following treatment with chemical carcinogenic agents. Cells from each stage can be isolated and maintained for study. During the steps of this progression, changes occur in the expression of many cell properties and their combined effect confers the capacity to form a progressively growing tumor. Since no single molecular alteration has been identified which, by itself, is sufficient for tumorigenicity, it is likely that the importance of each change lies in its relationship to the other changes which have occurred. In this study, each change in cell properties is viewed with regard to its appearance during the stages of pre-neoplastic progression and its relationship to other changes which have occurred.

The objectives of this project are (1) to identify, at the molecular level, changes occurring as cells progress through the stages of carcinogenesis; (2) to study the interrelationships among these changes for the purpose of identifying conditions of phenotypic expression necessary for tumor growth; and (3) to relate the essential phenotypic changes to specific alterations in regulatory mechanisms induced by chemical carcinogens. Emphasis is placed on those changes which occur on the cell surface or in the immediate extracellular area and which may be related to modification by tumorigenic cells of the hosts's capacity to inhibit nascent tumor formation. Specifically, the research focuses on alterations in the composition of the membrane and extracellular matrix, changes in susceptibility to natural host defenses, and disturbances in expression of proteolytic and protease inhibitory activities which may trigger a cascade of host responses either favorable or detrimental to the growtn of nascent tumor cells.

Methods Employed: Guinea pig cells treated with chemical carcinogens have been prepared and cells isolated at various stages along the pathway toward neoplastic transformation. These cultures, many of which have been preserved in liquid nitrogen, are a major source of material for this project. Cells are assessed for 1) tumorigenicity in syngeneic guinea pigs or other appropriate animals, 2) ability to elicit a sustained intradermal delayed type skin reaction in syngeneic immunologically compatible nonimmune animals, 3) sensitivity to the colony inhibitory effects of lymphotoxin, 4) expression of Forssman and other glycolipid antigens, 5) secretion of fibronectin, collagen and glycosaminoglycan matrix components and 6) production of enzymes involved in plasmin, thrombin, kinin and complement activation as well as the production of inhibitory molecules that function in the post-synthetic control of proteolytic enzyme activity. Lymphotoxin susceptibility is determined by inhibition of tumor cell colony formation. Surface antigen expression is quantitated with xenoantisera and radiolabeled *Staph* protein A. Enzyme activities and inhibitory factors are measured fluorometrically with synthetic substrates, in functional biological assays, and immunochemically with xenoantisera to the purified proteins.

Major Findings: The fibrinolytic activity of guinea pig cell lines and their sensitivity to guinea pig lymphotoxin vary directly with the number of cells required to produce a progressively growing tumor in syngeneic guinea pigs. These properties appear to be closely related to the tumorigenic capacity of the transformed cells and may be among the important determinants of tumor growth. The tumorigenic cells produce, in addition to the plasminogen activating enzymes, an acid stable inhibitor of plasmin activity. Tumorigenic cells, but not nontransformed or morphologically transformed nontumorigenic cells, produce a sustained intradermal tuberculin-like response in nonimmune syngeneic animals. This response too, appears to directly correlate with the tumorigenic capacity and may represent, like the sensitivity to lymphotoxin, an aspect of the host natural defenses against tumor cells. Examination of the skin induration after four days shows the tumorigenic cells to be present. Nontumorigenic cells are not evident at day four. Metabolic activity, but not proliferation, of the persisting tumorigenic cells is required to produce the response. Histologically, the persisting cells are confined to nests by a fibrous capsule surrounded by a dense infiltrate of mononuclear cells. All the tumorigenic cells form colonies in 0.3% agar, whereas the nontumorigenic cells did not express this property. Nontumorigenic cells exhibit a markedly greater expression of Forssman cell surface glycolipid than do tumorigenic cells. Fibronectin is produced in greater amounts by nontumorigenic cells, but this difference alone is neither large enough nor sufficiently consistent for tumorigenic cells to be distinguished from nontumorigenic cells. Likewise, unique, cell line distinctive, tumor-associated neoantigen expression is not a distinguishing feature since not all the tumorigenic cells express individually distinct surface antigens detectable by tumor transplantation protection tests, by delayed-type hypersensitivity skin tests, and by antisera from syngeneic and xenogeneic animals immunized with the tumorigenic cells.

Significance to Biomedical Research and the Program of the Institute: Many cell surface molecules or associated activities are altered on tumor cells. When these alterations occur as a consequence of chemical carcinogen treatment, they do not reflect the introduction of new genetic material, but rather are due to disturbances in the regulation of normal gene products or are due to a direct action of the chemical agent on structural or regulatory genes. Altered expression of properties associated with the cell surface and involved in interactions with the cell's environment are being studied with immunochemical and physical biochemical techniques capable of identifying and isolating specific molecular components that can be functionally, as well as physicochemically, identified and their expression related to changes in specific regulatory components. This work is being done using the guinea pig carcinogenesis model with its extended stages of carcinogenesis and the Syrian hamster model of carcinogenesis where the same progression occurs over a more condensed period. Investigations underway concern the role of carcinogen treatment in the immediate and heritable expression of cell surface neoantigens and the altered expression of normal cell surface components, the range of individuality found, and any repetitive occurrences which may indicate the limits of the variations generated and the role of specific interacting factors. Later work will approach the molecular nature of selected components and possible effects of their expression on the immunoregulatory mechanisms of the tumor-bearing host.

These studies will determine the importance of specific tumor cell surface and intracellular factors in enabling progressive in vivo growth of tumor cells. Identification of these factors and the mechanisms of their function and interrelations will provide insight into the necessary cellular changes which occur during carcinogenesis. Through this insight, points of intervention may be identified at which the carcinogenesis process may be halted or at which tumor growth may be reversed.

Proposed Course of the Project: Investigations will continue which focus on mechanisms through which cells acquire their ability to form progressively growing tumors. The current base of data will be expanded through the study of additional properties whose expression may be disturbed as a consequence of alterations in the genetic regulatory mechanisms, and the data will be extended to other cell lines and species. Those properties found to be most closely related to tumorigenicity will be further investigated to establish their role in the altered expression of other properties by tumorigenic cells and their influence on the host's immunological and inflammatory response mechanisms.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05206-01 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Normal and Malignantly Transformed Cell Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. P. Fuhrer	Expert Scientist	LB NCI
OTHER:	C. H. Evans	Chief, Tumor Biology Section	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biology, Carcinogenesis Intramural Program

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.25	0.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell surface membranes mediate interactions between cells, such as cell-cell recognition, adhesion, and alignment, that may depend upon highly organized molecular complexes within the membrane or exposed at the membrane inner or outer surface. Abnormal interactions between malignant and host cells or host effector mechanisms may be the result of compositional and/or structural abnormalities in the molecules that comprise the external architecture of membranes. Quantitative methods utilizing computer facilitated analyses of one and two dimensional IEF/SDS gel electrophoresis and HPLC have been established to characterize the protein, glycoprotein, lipid, and glycolipid components of normal and transformed cell membranes and to determine the molecular basis of membrane changes that may occur in response to the treatment of cells with chemical and physical carcinogens, co-carcinogens, modulators, lymphokines, and other immunobiological effectors. These studies have demonstrated that treatment with the lymphokine, lymphotoxin, stimulates the synthesis of high molecular weight membrane glycoproteins in normal cells and decreases incorporation of glucosamine into high molecular weight glycoproteins in malignantly transformed cells without significantly altering membrane protein composition in either cell type.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The primary objectives of this project are to identify changes in the molecular composition and/or structure of the membranes of normal and malignantly transformed cells that occur as a prerequisite to, or as a necessary accompanyment of, the transformed (neoplastic) state of a cell and to determine the molecular basis by which immunobiological modifiers interact with normal and transformed cells in order to effectuate control over the proliferation of neoplastic cells. Specific objectives include: (1) quantitative and qualitative characterization of membrane proteins, glycoproteins, lipids, and glycolipids to differentiate normal, preneoplastic, morphologically transformed, and tumorigenic cells; (2) demonstration of quantitative and qualitative compositional and structural changes in membranes that are associated with the progress of cells toward the neoplastic state; (3) quantitative determination of compositional and/or structural changes in membranes that occur in response to the treatment of cells with chemical carcinogens, UV and X-irradiation, tumor promoters and other co-carcinogens, lymphokines and other immunological effectors; and (4) isolation, quantitation, and structural analysis of cell surface antigens and receptors from normal and transformed cells. Particular emphasis is placed on the specific nature of structural alterations that occur in the oligosaccharide portions of glycoproteins derived from cells during their progress toward neoplasia following the initiation of carcinogen-induced transformation.

Methods Employed: This study utilizes Syrian hamster, guinea pig, and human normal, preneoplastic and neoplastic cells. Cells growing in culture are metabolically labeled with radioactive protein and carbohydrate precursors or externally labeled with ^{125}I or ^{3}H $NaBH_4$. Membranes or membrane extracts are prepared from labeled cells and are analyzed for relative composition and structure by one and two dimensional IEF/SDS gel electrophoresis, HPLC, TLC, and other analytical and preparative biochemical and physicochemical techniques. Isolation of specific cell surface antigens for comparative quantitation or structural analysis is accomplished by indirect immunoprecipitation through the use of monoclonal antibodies. Monoclonal antibodies are produced with specificities for unique transformed cell surface antigens, for antigens present on both normal and transformed cells, for antigens expressed by cells in response to treatment with carcinogens, cocarcinogens, lymphokines, and other factors, and for antigens expressed on fetal cells that cross-react with transformed cells. One and two dimensional gels are quantitated and compared by computer-assisted data acquisition systems using the NIH central computer. Preparation of unique cell populations and studies of the distribution of cell surface antigens are accomplished by combinations of electrophoretic, centrifugal, chromatographic and cytofluorographic techniques through the use of monoclonal antibodies reactive with cell surface determinants.

Major Findings: Major findings are currently limited to those derived from data describing the effects of lymphotoxin on the membranes of normal and transformed cells. Lymphotoxin is a lymphokine, non-antibody mediator of cellular immunity, that possesses the ability to inhibit carcinogenesis and modulate the growth of tumor cells.

1. Normal hamster embryo fibroblasts (HEC 2°) respond to lymphotoxin treatment by an increase in the synthesis of high molecular weight membrane glycoproteins. This increase is evident after 24 hours of lymphotoxin treatment and is proportional to the dose of lymphotoxin used. This is the first demonstration of a lymphotoxin-induced biophysicochemical alteration in normal cells that may be related to the anti-carcinogenic and tumor inhibitory activities of this lymphokine.

2. Lymphotoxin treatment results in decreased incorporation of glucosamine into high molecular weight glycoproteins in lymphotoxin sensitive transformed HEC. This response is also dependent on the dose of LT used and has been demonstrated in two different transformed HEC cell lines. It is qualitatively and quantitatively the opposite effect of that observed in lymphotoxin-treated non-transformed cells.

3. The differential synthesis of high MW glycoproteins in normal LT-treated cells and decrease in glycoprotein presence in transformed HEC occurs without significant changes in detectable protein composition of the membranes. This is of considerable interest as glycopeptides are an integral part of cell surface antigens and mono- and oligosaccharides constitute determinant features of cell membrane glycoproteins and glycolipids, some of which are known to be receptors for host immunobiological and other physiological regulatory interactions.

Significance to Biomedical Research and the Program of the Institute: Detailed molecular investigations of the membranes of normal and transformed cells will elucidate the structures and mechanisms through which the immune and other physiological systems of the host interact with normal, preneoplastic, and neoplastic cells in vivo. Systematic characterization of compositional and structural changes in the cell surface and/or its components that occur as a result of treatment with chemical and physical carcinogens, modulators, effectors, etc., will provide insight into the mechanism(s) responsible for neoplastic transformation and its control. Understanding the molecular mechanisms that underly neoplastic transformation and host control of cancer ultimately will provide the background for novel approaches to the detection, prevention and control of cancer.

Proposed Course: The initial phase of this project encompasses qualitative and quantitative evaluations of the structural components of the membranes of normal cells which will serve as a background for characterizations of membrane changes that occur during the transition of cells from the growth controlled to the neoplastic state. These studies are being conducted simultaneously with experiments designed to define and quantitate compositional and structural changes in membranes that occur in response to treatment of cells with carcinogens, co-carcinogens, and immunological effectors, specifically lymphokines. The preparation of monoclonal antibodies to normal, fetal and transformed cell antigens and to lymphotoxin currently in progress will be expanded into a major production effort for carcinogenesis-related hybridomas since monoclonal antibodies will be needed in later stages of the project for the sorting of cell populations, analyses of antigen distribution and turnover, isolation of transformed cell surface antigens for structural comparison to crossreacting normal and fetal antigens, and for the isolation and structural analysis of LT receptors. This biochemical and immunochemical approach, combined with computer-facilitated quantitation, will permit intensive analyses of the mechanisms of carcinogenesis and of the responses of cells to host effectors.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05207-01 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Carcinogen-induced Transformation of Mammary Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. W. Greiner	Staff Fellow	LB, NCI
OTHER:	C. H. Evans	Chief, Tumor Biology Section	LB, NCI
	J. A. DiPaolo	Chief, Laboratory of Biology	LB, NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Biology, Carcinogenesis Intramural Program

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 1.25	OTHER: 0.75

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An in vitro model system has been developed for the study of the interrelationships between differentiation and carcinogenesis in hormonally-responsive mammary epithelial cells. The epithelial cells lining the ducts and alveoli of the mammary glands of Sprague-Dawley rats are separated from stromal and fat cells by enzymatic digestion followed by density gradient centrifugation. These epithelial cells are susceptible to chemical carcinogens and proliferate when added to a hormone-supplemented medium. Whereas normal mammary epithelial cells have a finite life span of approximately 2-3 weeks, after carcinogen treatment cells can be subpassaged and cultured for three months. Carcinogen-treated mammary epithelial cells acquire anchorage independent growth, but thus far have not formed tumors when injected into nude mice.

Project No. Z01 CP 05207-01 LB

Project Description

Objective: The purposes of this project are 1) to elucidate the cellular and molecular events involved in the induction and development of breast cancer; 2) to relate carcinogenesis and differentiated function in hormonally-responsive mammary epithelial cells; 3) to develop markers for carcinogen-induced transformation of mammary epithelial cells; and 4) to identify regimens which can inhibit, reverse or retard the carcinogenic process in these target cells. The development of an in vitro model for the study of carcinogen-induced transformation of the epithelial cells derived from hormonally responsive Sprague-Dawley rat mammary glands is of paramount importance.

Methods Employed: While the fat and stromal cells constitute the majority of cells in the mammary glands, the biological function (i.e. lactation) of the endocrine-sensitive target organ is a property of the epithelial cells which line the ducts and alveoli. These cells are the presumed target cells for carcinogens for both the rat and human. Techniques have been developed for the routine isolation of epithelial cells from the rat mammary fat pad. The abdominal and inguinal fat pads are excised, enzymatically digested and the fat cells separated by differential centrifugation. Fibroblasts are separated from multicellular epithelial aggregates by gradient centrifugation. The epithelial nature of the isolated cells has been established by the presence of high-affinity steroid receptors, casein and α-lactalbumin production, presence of desmosomes and microvilli and the ability to form normal mammary architecture and carry out normal physiological functions when transplanted back into the cleared rat mammary fat pad. A hormone-supplemented medium has been developed for the mammary epithelial cell. Primary cultures of mammary epithelial cells with the enzymatic capacity for oxidative metabolism are exposed to chemical carcinogens. Neoplastic transformation is assessed by changes in the growth capabilities of these cells in vitro, induction of morphological changes, acquisition of anchorage independent growth and the tumorigenicity of carcinogen-treated cells by injecting into homozyous athymic nude mice and/or into the cleared fat pad of immunosuppressed weanling Sprague-Dawley rats.

Major Findings: Isolated rat mammary epithelial cells can be routinely grown in a hormone-supplemented medium consisting of cortisol, progesterone, 17β-estradiol, insulin and prolactin added at physiological concentrations. The cells can be maintained in primary culture for 2-3 weeks until they cease to divide. During this time in vitro rat mammary epithelial cells form multicellular hemicysts, often referred to as domes, which are considered an in vitro characteristic of epithelial cells. Carcinogen treatment of mammary epithelial cells evokes certain phenotypic changes which are not observed in the untreated, control cells. Rat mammary epithelial cells treated with DMBA or MNNG can be subpassaged after 2-3 weeks in culture. Furthermore, the carcinogen-treated cells have been maintained in vitro for up to 3 months during which time dome formation persists, indicating the differentiated epithelial characteristics of the cells. Therefore, carcinogen treatment imparts upon the mammary epithelial cells an extended life span not seen in control cells. In addition, carcinogen-treated cells also acquire the ability to form colonies in agar, a phenotypic property often associated with neoplastic transformation. However, cells isolated from agar colonies of rat mammary epithelial cells have a finite life span, and when injected into homozyous athymic nude mice, do not produce tumors.

Therefore, although carcinogen treatment induces changes in the cells' phenotypic characteristics (lifespan and anchorage independence) which are often correlated with tumorigenicity, the neoplastic transformation of these cells, as evidenced by tumor formation, is still lacking.

Significance to Biomedical Research and the Program of the Institute: Breast cancer is the most common neoplasm in women of the Western world and the pathogenesis of the disease remains unknown. The cause and growth of breast cancer has a strong genetic component. A major portion of that component may involve hormonal stimulation. Endocrine influences during early menarche, as well as late menopause, are associated with an increased risk of breast cancer. On the other hand, an early first, full-term pregnancy often protects against breast cancer. Specific markers (i.e. receptors) for endocrine-dependent growth are used for selecting breast cancer patients most likely to respond to endocrine intervention therapy.

To date, the best species in which to study hormonally influenced breast cancer is the rat. DMBA-induced mammary adenocarcinoma formation is under genetic control: i.e., varies form 100% in Sprague-Dawley to 0-10% in Long-Evans rats. Moreover, the induction by DMBA of mammary tumors in the Sprague-Dawley rats is strictly influenced by the endocrine state of the animal. Specifically, pregnancy and lactation impart a protection against DMBA-induced tumors, as do the removal of the ovaries and/or pituitary prior to carcinogen treatment. The ablation of endocrine organs during the growth of rat mammary tumors results in tumor regression.

Therefore, the similarities of genetic factors and requirements for continued endocrine stimulation establish a basis for using the rat as a prototype for studying human mammary carcinogenesis. The development of an in vitro transformation systems for mammary epithelial cells will provide an opportunity 1) to determine the mechanisms whereby chemical carcinogens interrupt normal mammary epithelial differentiation that results in tumor formation, 2) to determine the involvement of hormones in differentiation and carcinogenesis at the target cell level, 3) to develop specific markers for the transformation of mammary epithelial cells, and 4) to initiate concomitant study of in vitro transformation of human breast cells to establish a basis for extrapolation of data between the two species.

Proposed Course: Investigations will continue to define the requirements for an in vitro transformation system for rat mammary epithelial cells to serve as a model for the development of a human breast epithelial cell culture system. Immediate goals will be to explore the mechanisms whereby carcinogens induce an extended life span and anchorage independent growth in mammary epithelial cells, but not the ability to form tumors when injected in vivo. Possibly, new culture conditions may be required for the continued growth of carcinogen-transformed epithelial cells to permit completion of carcinogenesis. These conditions may include the addition of growth factors specific for nonmalignant and/or for neoplastic mammary epithelial cells.

Publications:

None

SUMMARY REPORT

LABORATORY OF CARCINOGEN METABOLISM

October 1, 1980 through September 30, 1981

The Laboratory of Carcinogen Metabolism (LCM) plans, develops and conducts a research program including (1) identification, chemical analysis, mode of formation, and metabolism of various classes of chemical carcinogens; (2) studies on the toxicology and metabolic pathways of carcinogens in selected animal systems and in relation to man; (3) correlation of chemical and toxicological data for the selection and screening of chemical carcinogens; and (4) development of biological and biochemical methods for the identification of reactive carcinogenic metabolites.

The Laboratory of Carcinogen Metabolism is involved to a minimal extent in the scientific direction of many collaborative projects at present. Although the major emphasis of the Laboratory of Carcinogen Metabolism is on various intramural research projects, personnel of the LCM are involved as consultants or advisors on various interagency, national and international activities in the area of chemical carcinogenesis and environmental carcinogens. They furnish advice to other government agencies, to the industrial or academic world and serve on various panels, boards or committees to deal with current problems relating to chemical carcinogens and laboratory safety. Staff members are also project monitors for various aspects of the program at FCRC.

Research projects in the Office of the Chief are mainly directed toward studies on the metabolism and mechanism of action of aromatic amines and dialkylnitrosamines. Physical-chemical differences between normal rat hepatocytes in culture and those which have been transformed by treatment with nitrosomethylurea are also under investigation.

With respect to the metabolism of dimethylnitrosamine, pyrazole was a potent inducer of dimethylnitrosamine demethylase. Electrophoretic separation of the liver microsomal proteins from pyrazole-induced rats demonstrated a large increase in a band at the 52,000 molecular weight region. Presumably, this band may represent the dimethylnitrosamine demethylase enyzme. On the other hand, in animals which had low levels of dimethylnitrosamine demethylase, due to partial hepatectomy, a single dose of dimethylnitrosamine led to an appreciable incidence of kidney tumors. Although the time required for development of these tumors was approximately one year, the tumors generally were very large. Thus, this system may serve as a useful animal model for study of kidney tumors.

Considerable progress has been made in separating and identifying some of the water-soluble metabolites of 2,4-diaminoanisole (2,4-DAA). One tentative metabolite is 3,8-diamino-2,9-dimethoxybenzo[c]cinnoline, which indicates various hydroxylaminoanisoles were possible intermediates. This type of structure is relatively unusual as a metabolite of an aromatic amine. Since there are no thorough previous studies on aromatic diamines, it remains to be determined whether this represents a usual or unusual metabolic pathway for diamines. Other metabolites tentatively identified are 2-acetylamino-5-hydroxy-p-anisidine, and 4-acetylamino-2-aminophenol. NMR comparisons allowed a definitive assignment of the structure of 4-(glycolamido)-2-aminoanisole, another water-soluble metabolite of 2,4-DAA.

Rat hepatocytes transformed to a malignant state by treatment in culture with nitrosomethylurea showed far fewer mitochondria than control hepatocytes, lack of the Golgi apparatus, and dispersed chromatin in the nucleus versus the condensed chromatin of the control cells. It was possible to grow both the control and transformed cells on microspheres; on this type of surface, the cells assumed the typical cuboidal shape of hepatic cells rather than the squamous appearance when grown on a flat surface. Lactate dehydrogenase (LDH) levels were four times greater in transformed cells than in control cells; as a corollary there was four times as much lactate in the medium from transformed cells. Likewise, pH values in transformed cells were in the range of 6.6, while in control cells the pH remained above 7. Other results indicated that the control LDH was in a phosphorylated state but that LDH from transformed cells was not.

Mutagenicity studies on urines of rats given various carcinogens were used as a quick means of following the effects of inhibitors or enhancers of carcinogens. Thus, feeding acetanilide increased the number of mutagenic revertants over that from N-2-fluorenylacetamide (FAA) alone; non-antagonists of FAA did not increase mutagenic activity. Feeding tyrosine increased the mutagenicity of urine from 2,4-DAA-treated rats. Thus, mutagenicity indicated the situations where the rate of metabolizing and excreting 2,4-DAA was increased.

Analytical Chemistry Section - Conducts independent research on the organic, analytical, and biological chemistry of nitrosamines and related compounds and collaborates with other members of the Carcinogenesis staff in matters requiring chemical expertise, especially in problems of identifying nonpolymeric organic molecules. Activities for the past year have focused on two main areas.

N-Nitroso Compounds. (a) A strategy by which nitrosamine contamination of cutting fluids can be reduced has been inferred from studies of the various chemical and physical factors contributing to such contamination. One step, replacement of the secondary and tertiary amines normally used in such products by suitable primary amines, is of potentially general applicability in eliminating N-nitroso carcinogens from other consumer products and workplace environments, including cosmetics and hydraulic fluids. (b) The goal of hazard control in the carcinogenesis research laboratory has been advanced by the discovery that nickel-aluminum alloy reduces nitrosamines quantitatively to the corresponding amines in a variety of media. This procedure may be a generally useful waste treatment method for disposal of most nitrogen-containing carcinogens. (c) A trapping agent previously recommended for probing biological methylation by carcinogens *in vitro* has been found to be susceptible to extensive complications due to artefact formation. (d) The barrier to internal rotation in several nitrosamines has been studied by nuclear magnetic resonance spectrometry, and some previously undocumented conformational effects have been recognized using x-ray crystallographic methods.

Mass Spectrometry and Nuclear Magnetic Resonance. The instrumentation laboratory carries out independent research and, in addition, collaborates with other investigators on carcinogenesis-related problems in which these analytical methodologies can be profitably employed. (a) Most carcinogenic N-nitrosodialkylnitrosamines are well suited for identification by mass spectrometry, which is still the technique of choice for positively confirming suspected nitrosamine contamination in the environment. To increase the predictive value of the method for new compounds, the electron impact induced fragmentation behavior or selected dialkylnitrosamines is being studied using deuterium labeled analogs.

(b) Spectral studies are continuing on the acetonide derivatives of di- and trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Both the spectral studies and the isotope exchange experiments on these compounds give valuable information about the special lability of substituents attached at carbon 10 of the tetrahydrobenzo(a)pyrene molecule. (c) In supporting projects within the Laboratory, spectral measurements, including ^{13}C NMR, on a number of synthetic intermediates and on metabolites from 2,4-diaminotoluene and 2,4-diaminoanisole have facilitated chemical structure determinations in this series. (d) Efforts are being continued in the analysis of bioactive materials that may play a role in cancer cause and prevention studies. Collaborative work aimed at elucidating the mode of action of retinoids led to the structural determination of several in vivo and in vitro metabolites of retinoic acid. Structural studies were also performed on the antitumor antibiotics Gilvocarcin V and Chrysomicin.

Nutrition and Metabolism Section - Plans, develops and conducts research on (1) the effects of dietary constituents on the activities of various classes of chemical carcinogens, (2) the metabolism of dietary components known to modify the carcinogenic process, and (3) the correlation of chemical and toxicological data for the identification of chemical carcinogens. The Section has focused its interest on the role of the lipotropes methionine, choline, vitamin B_{12} and folic acid in chemical carcinogenesis. The growth constants in methionine-deficient, homocysteine-supplemented medium of rat liver epithelial cells were shown to be a linear function of their intracellular levels of methyltetrahydrofolate:homocysteine methyltransferase. No significant differences were observed between the growth constants of transformed cells and those of normal cells in the homocysteine-supplemented medium. The growth constants of normal and transformed hepatocytes bore a sigmoidal, logarithmic relationship to the methionine concentration of the medium. Methylcobalamin stimulated the growth rates of both normal transformed lines on homocysteine-containing medium. Studies are currently underway comparing the carcinogenicity of ethionine in rats, hamsters and three strains of mice. The administration of dietary ethionine, like that of methyl-deficient diets, led to decreased hepatic contents of S-adenosylmethionine (SAM) and to increased levels of ornithine decarboxylase (ODC); the levels of ODC were inversely proportional to those of SAM. Hepatic SAM levels were also lowered by the chronic administration of the liver tumor promoters DDT and phenobarbital. These results are consistent with the hypothesis that methyl insufficiency promotes liver carcinogenesis. Finally, the isoacceptor $tRNA^{Met}$ has been shown to use ethionine as a substrate in vitro and to transfer the ethionine in protein biosynthesis.

The production of lung adenomas in strain A mice by nickel acetate and lead subacetate was inhibited by the simultaneous administration of calcium and magnesium acetates. Similarly, calcium inhibited the block by cadmium of the thymidine incorporation into the DNA of liver cells. The possible antagonism between divalent metal carcinogens and the essential divalent cations are being extended to include testicular carcinogenesis with cadmium.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04493-04 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Physico-chemical Studies of Differences between Normal and Tumor-producing Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ann E. Kaplan	Chemist	LCM NCI
OTHER:	Margaret R. Bunow	Expert	LCM NCI
	Nadine C. Chien	Biologist	LCM NCI
	Mary J. Wilson	Chemist	LCM NCI
	Tommie S. Tralka	Biologist, Electron Microscopist	LP NCI
	Barry Bunow	Senior Staff Fellow	LAS CR

COOPERATING UNITS (if any) Dr. H. Amos, Department of Microbiology, Harvard Medical School, Boston, MA; Dr. S. A. Margolis, National Bureau of Standards; Prof. M. Cassidy, George Washington University Medical Center, Washington, D.C.; and Dr. Mahlon Freeman, Genetics Screening and Counseling Center, Denton, TX.

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	2.1	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Physico-chemical studies of control (TRL 12-13) and nitrosomethylurea-transformed (NMU-3) hepatocyte lines include: 1) Morphology - change in cell shape from squamous to cuboidal when grown on microspheres; marked output of fibronectin from TRL 12-13 cells observed by scanning electron microscopy. Both cell lines seem to prefer microspheres to flat surfaces for growth. 2) Lactate dehydrogenase (LDH) and lactic acid production - LDH enzyme stabilized with proteolytic inhibitors responds differently to inhibitor-free NADH. Molecular differences are observed by gel electrophoresis and isoelectric focusing; exposure of TRL 12-13 to alkaline phosphatase results in conversion to NMU-3 type LDH, implying phosphorylated sites on control enzyme. Spectrophotometric analysis of internal pH demonstrates a four-fold higher rate of acid production in transformed cells, with 75% of the glucose in the growth medium being converted to lactic acid.

Project Description

Objectives: Tumor cells from clinical and experimental sources secrete increased amounts of lactic acid. Much of the potential chemical energy from carbohydrate is exported from the cell with incomplete utilization. In patients, this wasteful pathway leads to cachexia.

The immediate objectives are to identify the molecular basis for the shift to this energy-wasting pathway. For close relevance to human cancer, where over 90% of tumors are identified as carcinomas, we studied an epithelial cell line which was chemically transformed in vitro and produces carcinomas in vivo. This cell line has an elevated output of lactic acid and thus provides the opportunity to study the balance between lactic acid output and the molecular properties of the enzyme LDH. Although the general appearance of the transformed cell is unchanged, we are studying the ultrastructure of the transformed cell. Long-range objectives are to identify changes accompanying transformation which will be useful as a screen for chemical carcinogens and to consider methods for tumor cell destruction, based on the differences identified in the bioenergetic system in transformed cells.

Methods Employed: Physico-chemical methods employed in this study of control and chemically transformed hepatocytes include: 1) Morphological comparison by scanning and transmission electron microscopy; 2) studies of LDH from hepatocyte lines and fibroblast lines using kinetic analysis with the DASAR System using On-Line (computer) modelling systems for comparison of data, identification of molecular differences by gel electrophoresis and isoelectric focusing, including application of the latter to evaluate LDH modification after exposure to alkaline phosphatase; 3) analysis of lactate production relative to changes in pH in cytosol and medium by incorporation of 6-carboxyfluorescein, a pH sensitive indicator, into cells and detecting pH changes by direct measurements in cell monolayers by split-beam and dual-wavelength spectrophotometry, evaluation of rate of lactate production by drop in intracellular block induced by quercetin, enzymatic analysis of lactate in medium. Development of new methods for purification of LDH.

Major Findings: 1) Ultrastructural morphology. Both cell lines grow in monolayers and differ very little in appearance by light microscopy. For transmission electron microscopy the cells were fixed directly on the growing surface at confluence and 48 hours later. All characteristics of the mature hepatocyte appear in the control cell line, including polarity of distribution of intracellular elements with rough endoplasmic reticulum, Golgi apparatus, and mitochondria separate from dispersed polyribosomes. The nucleus had condensed chromatin with somewhat submerged nucleoli and a heavily marginated nuclear membrane. The plasma membrane shows rich development of microvilli, pinocytotic vesicles and coated vesicles. These cells secrete fibronectin and tend to form intercellular canaliculi.

The transformed cell, NMU-3, has the appearance of a "blast" cell with fewer intracellular organelles, little rough endoplasmic reticulum, far fewer mitochondria than in the control cell at confluence, and comparatively defective mitochondria with incomplete cristae and a moth-eaten matrix. After 48 hours the mitochondria of the control cells increase significantly, but the transformed cells never reach the number observed in the control cells at confluence. These cells also lack any Golgi apparatus. Polyribosomes are dispersed throughout the cell. The nucleus has finely dispersed chromatin, a very prominent nucleoli, and a very thinly marginated membrane. The plasma membrane is linear and lacks the microstructure of the TRL 12-13 cell.

In an effort to increase the output of cells for enzyme studies, we succeeded in growing both cell lines on two types of microspheres. These are among the first epithelial lines, especially hepatocytes, thus cultured. Unexpectedly, scanning electron microscopy showed hepatocytes grown on flat surfaces have the appearance of squamous cells although their intracellular structure is that of hepatocytes. However, cells grown on microspheres take on the typical cuboidal appearance of hepatic cells. They still grow as monolayers on these surfaces, and both cell lines appear similar. With microspheres which adhere to the growing surface, the control cells secrete fibronectin, but the transformed cells do not. With the non-adhering microspheres, fibronectin cannot be detected. Both cell lines appear to prefer growing on the microspheres rather than the flat surfaces. Since the microsphere method permits more cells to be produced more quickly, this may be the preferred means of growing these cell lines.

Transmission electron microscopic studies of cells grown on microspheres are in progress to see if the intracellular structure resembled that of the squamous type cells.

2) Lactate dehydrogenase (LDH). LDH extracted from TRL 12-13 and NMU-3 cells is now stabilized through the addition of four anti-proteolytic factors. Results obtained in this preparation confirm that there is four times as much LDH in NMU-3 compared with TRL 12-13 and that kinetic changes with transformation lead to a loss of inhibition to NAD+. The combined findings suggest that lactate formation is markedly facilitated in transformed cells.

Qualitative similarities exist in the two LDH preparations but they differ quantitatively -- gel electrophoresis reveals a dominant band of LDH-4 in TRL 12-13 with a lesser amount of LDH-5. The reverse is true of LDH isozymes from NMU-3. These isozymes characterize liver cells. Treatment of the isozyme mixture from NMU-3 with alkaline phosphatase produces no changes and the enzyme remains stable for at least two hours at 37°. Treatment of the isozymes from TRL 12-13 under the same conditions results in the electrophoretic pattern identified with the NMU-3 cells but with no loss of enzyme activity. These results imply phosphorylated state(s) LDH in the control cell, but not in the transformed cell.

This is further suggested by the distribution of isoelectric points for the two LDH preparations. With isoelectric focusing, the same bands are found in both cell extracts; but quantitatively the majority of the material from TRL 12-13 equilibrates below pH 6, whereas the majority of the enzyme from NMU-3 migrates to a pH zone above 8.

These results, plus the observed deficiency in mitochondria in the NMU-3 cell, suggest a deficiency in phosphorylating capability in the chemically transformed cell.

3) Lactic acid formation and intra- and extracellular pH changes. The observed acidification of the medium of NMU-3 cells strongly suggests increased lactic acid production. We find that four times as much lactate (25 mM) accumulates in the NMU-3 medium during growth. At confluence, the rate of lactate formation is three times that of TRL 12-13. These differences parallel the four-fold increase in LDH in NMU-3. The results indicate a very inefficient use of energy sources by these cells, most of the glucose being lost to lactate formation.

Intracellular changes in the dye 6-carboxyfluorescein, taken into a monolayer of cells, permit direct spectrophotometric analysis which show that the intra- and extracellular pH of the TRL 12-13 cells remains well above 7. NMU-3 cells accumulate lactate which equilibrates with the external medium, resulting in pH values as low as 6.6 as lactate moves across the membrane with H+ ion. The kinetics of this transport in both cell lines are similar, with a half time of 25-30 seconds at 27°C. Quercetin, a bioflavenoid which inhibits lactate transport, inhibits lactic acid export, but not import in both cell lines. Thus, it leads to a depression in intracellular pH. Pyruvate transport is also blocked by quercetin in both cell lines.

Thus the transport mechanism appears to be the same in both cells but the synthesis of lactate in NMU-3 shows a four-fold increase, in line with the increase in total enzyme units. This is in line with other medium to slow growing tumor cells, even though the NMU-3 or TRL 12-13 cells have the same appearance.

Significance to Biomedical Research and the Program of the Institute: The results demonstrate a deficiency in the ability of the transformed cell to generate chemical energy by the usual enzyme pathways -- thus the imbalanced production of lactic acid from glucose, a wasteful pathway in terms of the energy lost in this biochemical pathway. This imbalance would be expected on the basis of the transmission electron microscopy findings which identify the transformed cells as being deficient in total mitochondria and having defective mitochondria. Furthermore, this deficiency in the production of chemical energy comes from the alkaline phosphatase sensitivity of the LDH from control cells, but not transformed cells. The latter may already lack phosphate groups due to a fundamental deficiency of phosphorylating potential. Thus, a clearer understanding of the energy deficit evolves so that even the altered kinetic behavior of LDH may be the result of loss of molecular controls through deficiencies in phosphorylation capability.

As we identify enzyme changes in the transformed cell, the possibility increases of identifying the transformed cell independent of gross morphological changes, in both experimental and clinical material, as well as the development of more rapid methods of screening for chemical carcinogens through the mammalian cell systems. Finally, more detailed molecular understanding enhances development of antitumor agents based on defects in the bioenergetic system in such cells.

Proposed Course: Purify LDH from control and transformed cells to evaluate the kinetic behavior of the major components in the transformed versus the control cells. Evaluate the separated components for sensitivity to alkaline phosphatase. Continue collaborative studies to compare LDH from control 3T3 cells and polyoma-transformed 3T3 cells with respect to electrophoretic behavior, response to alkaline phosphatase, and kinetic behavior. Complete studies of changes in morphology of hepatocyte lines as a result of growth on microspheres. Complete calculations for lactic acid changes with transformation. Complete kinetic analysis of purified LDH from LDH-1 from red blood cells of patient with genetic defect in LDH.

Publications:

Kaplan, A. E., Weiss, E. R., Byrne, S. T., El-Torkey, N. B. and Margolis, S. A.: Purified reduced nicotinamide adenine nucleotide response to lactate dehydrogenase from three cells. Science 212: 553-554, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04510-08 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Environmental and Genetic Factors in Digestive System Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard S. Yamamoto	Chemist	LCM NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

BALB/cN strain mice are quite susceptible to 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) carcinogenesis. However, C57Bl/6N strain mice are resistant to DMH, but not to AOM carcinogenesis. There is a sex difference in the response to the colon carcinogens, DMH and AOM. Estrogens play an important role in the shift of target organ from colon to the kidney, mediated by resistance of the female to DMH.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To determine interrelationships of environmental and genetic factors in the development of gastrointestinal tract carcinogenesis, particularly the role of hormones and dietary factors.

Methods Employed: Different strains of mice are tested for susceptibility or resistance to dimethylnitrosamine (DMH) and azoxymethane (AOM) colon carcinogenesis. BALB/cN and C57Bl/6N mice show large differences in response to colon carcinogens. Hormones also play an important role with these carcinogens. Male and female rats are castrated and implanted with estrogens. These rats are administered AOM, s.c., to develop tumors and their responses are studied. The response of the kidney to tumorigenicity is monitored.

Major Findings: BALB/cN strain mice, like the ICR strain, were very susceptible to DMH and AOM carcinogenesis. The lower colon developed many polyps (similar to human polyposis) which evolved into adenocarcinoma. C57Bl/6N mice were resistant to DMH carcinogenesis, but susceptible to AOM carcinogenesis. AOM was toxic to this strain at the equivalent effective dose for DMH carcinogenicity (0.2 mmol level), but carcinogenic at the lower (0.1 mmol) level.

With F344 (Fischer) strain rats, the females were resistant to DMH colon carcinogenicity but susceptible to AOM, with the additional development of kidney adenocarcinoma. Male rats responded to DMH and AOM with colon tumors, but failed to develop kidney adenocarcinoma even when survival time was extended and a larger dose was administered. Castrated male rats with implanted estrogen responded like female rats. Both 17-β-estradiol (E_2) and diethylstilbestrol (DES) were effective in producing kidney tumors in castrated male rats treated with AOM. Implantation of estrogens to enhance kidney tumorigenesis was effective only when administered with AOM and not before or after.

Significance to Biomedical Research and the Program of the Institute: The high morbidity and increasing mortality from gastrointestinal cancer is a major health problem in the United States. The sex difference in susceptibility to most gastrointestinal tumors and other non-endocrine target organ tumors shows an imbalance of hormone levels and more importantly hormone receptor levels may be a determinant. The development of a model where the target organ is changed by hormonal manipulation is important in studying carcinogenesis and also to study the role of hormone receptors in carcinogenesis.

Proposed Course: Further studies of the effect hormonal factors have in conjunction with gastrointestinal tract cancer and the study of the presence of estrogen receptors and its involvement in kidney carcinogenesis will be undertaken.

Publications:

Nishinaga, K. and Yamamoto, R. S.: Electrophoretic characterization of mouse liver polyribosomes. Anal. Biochem. 108: 185-189, 1980.

Nawata, H., Yamamoto, R. S. and Poirier, L. A.: An inverse correlation between uterine and ovarian levels of ornithine decarboxylase and S-adenosylmethionine decarboxylase in the rat. Proc. Soc. Exp. Biol. Med. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04542-09 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Chemistry of N-Nitroso Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Larry Keefer	Chief, Analytical Chemistry Section	LCM NCI
OTHER:	Peter Roller	Chemist	LCM NCI
	Thomsen Hansen	Staff Fellow	LCM NCI
	Allison Dorries	Physical Science Aid	LCM NCI

COOPERATING UNITS (if any) Drs. J. Fanning and J. Fesce, Clemson U.; Drs. R. Evarts & R. Angeles, NCI; Dr. A. Croisy, INSERM; Dr. C. Day, Crystalytics Co.; Dr. R. Loeppky, U. of Missouri; Drs. C. Michejda, M. Kroeger-Koepke, G. Lunn & E. B. Sansone, FCRC; and Dr. W. Gaffield, W. Regional Research Laboratory, USDA.

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Analytical Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Data concerning the chemical and physical properties of carcinogenic N-nitroso compounds are collected. Specific emphases include: (a) the preparation of novel nitrosamines and their derivatives for chemical and biological studies; (b) the biological chemistry of nitrosamines, both in vivo and in microsomal systems, with special attention to the rate modifying effects of deuterium substitution; (c) mechanistic studies of N-nitrosation reactions of environmental interest, particularly those promoted by electrophilic species, including transition metal complexes. Possible implications of this work with respect to the overall goal of human cancer prevention will be considered.

Project Description

Objectives: (1) To learn about mechanisms of nitrosamine formation to permit development of strategies for preventing environmental contamination by these compounds. (2) To gather information on the chemistry of nitrosamine destruction, so that procedures for intercepting them before human exposure can occur may be devised. (3) To study the interactions between N-nitroso compounds and organisms exposed to them, with the aim of inferring ways of protecting victims of unavoidable nitrosamine exposure from their carcinogenic effects. (4) To characterize the fundamental physical and chemical properties of the carcinogenic N-nitroso compounds as a means of contributing to the general fund of knowledge about such materials.

Methods Employed: In addition to the standard methods of synthetic and mechanistic chemistry employed in these studies, the Section now has acquired a detector system known as a Thermal Energy Analyzer, which is highly selective for the nitrosamino moiety. This technique is expected to help us greatly in problems requiring sensitive detection of N-nitroso compounds.

Major Findings: (1) In our nitrosamine formation studies, we have compiled extensive data on the accumulation of N-nitrosodiethanolamine (NDElA) in a common commercial metalworking formulation containing both alkanolamines and nitrite. Heating and acidification produced the most dramatic increases in NDElA contamination, but the tertiary and secondary alkanolamines proved to be inherently prone to surprisingly rapid N-nitrosation even in the normal, alkaline control reaction mixture stored at room temperature. In addition, the catalytic effects of certain transition metal complex ions and preservative agents found in cutting fluids were also potentially important. Nitrite-free products also produced nitrosamines when exposed to a common air pollutant, NO_x, partially offsetting the advantages of a nitrosamine reduction approach already receiving some use which involves the omission of nitrite from commercial formulations. Replacement of the amines normally used in these products by a primary amine analog, glycolamine, led to dramatic reductions of detectable nitrosamine yield; this primary amine substitution strategy, together with several other recommendations which can be inferred from these data, could ultimately lead to virtual elimination of nitrosamine contamination in a variety of consumer products and workplace environments, including in cosmetics and certain hydraulic fluids, as well as in the machine shop.

Basic research on nitrosamine-forming mechanisms has also been pursued. Thialdine and 3,3,4,4-tetrafluoropyrrolidine, two weakly basic amines, proved totally unreactive toward suspended sodium nitrite in methylene chloride solution. In addition, an X-ray crystallographic study of a compound formed by reaction of nitric oxide and oxygen with the μ-oxo dimer of ethylenediamine(salicylideneiminato) iron (III) suggested that this nitrosating agent, which is active in lipophilic media, is a nitrato complex; if this conclusion can be confirmed, it would provide unprecedented evidence that coordinated nitrate can serve directly as a reagent for converting secondary amines to the carcinogenic N-nitroso derivatives. Possible mechanisms of nitrosamine formation in pesticides have been reviewed. Finally, we have confirmed that the formation of nitrosamines from the diethylamine-nitric oxide adduct, a compound type last worked on by Drago and coworkers before nitrosamines were generally recognized as carcinogens, occurs only in the presence of oxygen, while in a non-oxidative atmosphere, dissociation to the amine and nitric oxide takes place; solution NMR data on the Drago complex have proven to be more nearly consistent with the assigned structure than were previously reported spectra, which appear to have been obtained on decomposed samples.

(2) Studies of nitrosamine destruction methods have revealed that nickel/aluminum alloy is a convenient reagent for degrading laboratory wastes containing not only a variety of carcinogenic N-nitroso compounds, but also nitramines, hydrazines, and N-hydroxy compounds. The method yields the presumably non-toxic parent amine as the ultimate product in every case studied thus far, and thus appears to be a substantial improvement over previously recommended procedures using aluminum foil as reductant, which led to the carcinogenic 1,1-dialkylhydrazines as the major product. Dimethylnitrosamine has been detoxified according to this method in methanol, methylene chloride and hexane, as well as in aqueous solution. The procedure holds considerable promise as a safe, inexpensive, efficient, reliable and general procedure for degrading nitrosamines, as well as a variety of other toxic nitrogen compounds employed in carcinogenesis research.

(3) A nucleophilic sulfur compound, 3,4-dichlorothiophenol, previously recommended as an in vitro trap for biological alkylating agents, has been studied in detail as a substrate for methylation resulting from metabolism of dimethylnitrosamine by the S-9 fraction of rat liver preparations. The method is susceptible to such extensive artifactual methyl thioether formation that yields up to 1000-fold greater than expected have been observed; the artifactual formation apparently occurred on the gas chromatographic column used for analysis, where an as yet unidentified non-volatile biological methylating agent injected during one analysis remains on the column to react with the sodium salt of the thiophenol injected in a subsequent analysis of the Na_2CO_3-extracted incubation mixtures.

(4) Several interesting results have been found in our studies of the fundamental chemical and physical properties of the carcinogen nitrosamines. The N-nitroso derivative of thialdine, a compound known since 1847, but which has only recently been recognized as a foodstuff component, was shown by X-ray diffraction to contain equatorial methyl groups flanking the nitrosamino group on both sides, in contrast to other nitrosamines whose conformations have been studied, all of which have been shown to possess axial substituents at the alpha positions. The steric crowding associated with this unusual stereochemistry can be credited with producing the first evidence of non-planarity in a nitrosamino group, which is both twisted about the N-N bond and displaced from the C-N-C place in N-nitrosothialdine. Studies of the barriers to internal rotation about the N-N bond by NMR methods have been performed on this compound, showing a low activation energy presumably attributable to the electronegativity of the two alpha sulfur atoms, as well as to the steric crowding identified in the X-ray studies, and on several other compounds as well.

Publications:

Croisy, A. F., Fanning, J. C., Keefer, L. K., Slavin, B. W. and Uhm, S.-J.: Metal complexes as promoters of N-nitrosation reactions: A progress report. In Walker, E. A., Griciute, L., Castegnaro, M. and Börzsönyi, M. (Eds.): N-Nitroso Compounds: Analysis, Formation and Occurrence. IARC Scientific Publication No. 31. International Agency for Research on Cancer, Lyon, 1980, pp. 83-94.

Roller, P. P., Keefer, L. K. and Slavin, B. W.: Inhibitory agents and chemical mechanisms in the dihalomethane-mediated nitrosation of amines with solid nitrite. In Walker, E. A., Griciute, L., Castegnaro, M. and Börzsönyi, M. (Eds.): N-Nitroso Compounds: Analysis, Formation and Occurrence. IARC Scientific Publication No. 31. International Agency for Research on Cancer, Lyon, 1980, pp. 119-128.

Walters, C. L., Hart, R. J., Keefer, L. K. and Newberne, P. M.: The sequential determination of nitrite, N-nitroso compounds and nitrate and its application. In Walker, E. A., Griciute, L., Castegnaro, M. and Börzsönyi, M. (Eds.): N-Nitroso Compounds: Analysis, Formation and Occurrence. IARC Scientific Publication No. 31. International Agency for Research on Cancer, Lyon, 1980, pp. 389-402.

Gaffield, W., Lundin, R. and Keefer, L. K.: Chiroptical properties of N-nitrosopyrrolidines and N-nitrosamino acids: Implications for the nitrosamine sector rule. Tetrahedron (in press).

Lunn, G., Sansone, E. B. and Keefer, L. K.: Reductive destruction of N-nitrosodimethylamine. Fd Cosmet. Toxic. (in press).

Roller, P. P., Keefer, L. K., Bradford, W. W. and Reist, E.J.: Synthesis, analysis, and stability studies of ^{14}C-methyl(acetoxymethyl) nitrosamine. J. Label. Compounds Radiopharmac. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04580-07 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Role of Lipotropes in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lionel A. Poirier	Chief, Nutrition & Metabolism Section	LCM NCI
OTHER:	Juanita Brown	Biological Laboratory Technician	LCM NCI
	Camille Hyde	Staff Fellow	LCM NCI
	Lawrence Lanier	Biological Laboratory Technician	LCM NCI
	Yves Mikol	Staff Fellow	LCM NCI
	Mary J. Wilson	Chemist	LCM NCI
	Nina V. Myasishcheva	Guest Worker	LCM NCI
	Dolph L. Hatfield	Molecular Biologist	LCM NCI

COOPERATING UNITS (if any)
Dr. Martin Wenk, Microbiological Associates, Bethesda, MD; Dr. Jerrold Ward, Tumor Pathology Branch, NCI; Dr. Allen Manus, Litton-Bionetics, Inc.; Mr. Don Creasia, FCRC.

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	2.7	0.9

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms behind the alteration of chemical carcinogenesis by the dietary lipotropes, choline, methionine, folic acid and vitamin B_{12} have been studied. The metabolism and carcinogenic activity of ethionine in different species is being compared. Ornithine decarboxylase, a marker of tumor promotion and a precursor enzyme for the polyamines, is used to study the possible role of methyl insufficiency in tumor promotion. The levels of S-adenosylmethionine in the livers of animals treated with hepatocarcinogens and liver tumor promoters are being determined. The results obtained to date are consistent with the hypothesis that methyl insufficiency promotes hepatocarcinogenesis.

PHS-6040
(Rev. 2-81)

Z01 CP 04580-07 LCM

Project Description

Objectives: The dietary lipotropes methionine, choline, and vitamin B_{12} significantly modify the production of tumors by certain chemical carcinogens. The extent of and the mechanisms behind these effects are being investigated. The effects of the lipotropes on chemical carcinogenesis are under investigation. In addition, the mutual metabolic interactions of the lipotropes and the chemical carcinogens are to be determined.

Methods Employed: The ability of ethionine to be incorporated into eukaryotic protein was examined using a wheat germ extract, cell free protein synthesizing system. The carcinogenic activities of several standard carcinogens were compared in rats fed defined diets. S-Adenosylmethionine and S-adenosylethionine are determined using isotope-dilution techniques and thin layer chromatography followed by spectrophotometric assay. The nutritional requirements of normal and transformed liver epithelial cells were determined in tissue culture systems developed in this laboratory.

Major Findings: Methylcobalamin stimulated the growth of two normal and three transformed rat liver cell lines grown in methionine-deficient, homocysteine-supplemented medium. The chronic administration of methyl-deficient, amino acid-defined diets led to increased hepatic levels of ornithine decarboxylase (a marker of tumor promotion), S-adenosylmethionine decarboxylase, and thymidine kinase; the levels of these enzymes tended to be inversely correlated to the methyl content of the diet. Ethionine, which is carcinogenic to rat liver, but noncarcinogenic in hamsters, formed higher hepatic levels of S-adenosylethionine in rats than in hamsters. Ethionine was recognized by rat liver methionyl-tRNA synthetase, aminoacylated to methionine tRNA isoacceptors and transferred from $tRNA^{Met}_{m}$ to protein at a rate comparable to that of methionine. The chronic administration of ethionine to rats increased the hepatic levels of ornithine decarboxylase as an inverse function of the decreasing contents of S-adenosylmethionine. The chronic administration of the liver tumor promoters phenobarbital and DDT led to decreased hepatic contents of S-adenosylmethionine.

Significance to Biomedical Research and the Program of the Institute: One of the basic aims of the National Cancer Institute is the prevention of cancer by a delineation of the mechanism by which carcinogens induce tumors. The aim of these studies is to determine whether methyl insufficiency is a promoter of liver tumor formation.

Proposed Course: Carcinogenesis by standard carcinogens in animals fed chemically defined diets will be determined. The carcinogenicity and metabolism of ethionine in different species will be completed. The effects of methionine deficiency on tumor promotion will be determined. The metabolism of selenium and the carcinogenicity of its metabolites will be examined.

Publications:

Hyde, C. L., Rusten, R. and Poirier, L. A.: A thin-layer chromatographic method for the quantitative separation and estimation of S-adenosylmethionine and S-adenosylethionine in rat liver. Anal. Biochem. 106: 35-42, 1980.

Mikol, Y. B., Roux, R., Decloitre, F. and Fournier, E. P.: Liver enzymatic induction in lindane- and captan-treated rats. Comparison with phenobarbital- and methylcholanthrene-treated rats. *Fd Cosmet. Toxicol.* 18: 377-382, 1980.

Poirier, L. A. and Wilson, M. J.: The elevated requirement for methionine by transformed rat liver epithelial cells in vitro. *Annals NY Acad. Sci.* 349: 283-293, 1980.

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Wilson, M. J., Hatfield, D. L. and Poirier, L. A.: Aminoacylation of ethionine to rat liver $tRNA^{Met}$ and its incorporation into protein. *FEBS Letters* (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04581-06 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Applications of NMR and Mass Spectrometry to Problems in Carcinogenesis Research

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter P. Roller	Chemist	LCM NCI
OTHER:	Larry K. Keefer	Chief, Analytical Chemistry Section	LCM NCI
	J. Richard Miller	Chemist	LCM NCI
	Blake W. Slavin	Physical Science Aid	LCM NCI
	Elizabeth K. Weisburger	Chief	LCM NCI
	Preston H. Grantham	Chemist	LCM NCI
	Timothy Benjamin	Chemist	LCM NCI
	Robert C. Atkins	Guest Worker	LCM NCI

COOPERATING UNITS (if any) M. B. Sporn, Chief, LC, NCI; C. A. Frolik, Chemist, LC, NCI; L. M. DeLuca, Chief, Differential Control, LEP, NCI; T. Wei, Fermentation Lab., FCRC; U. Weiss, LCP, NIAMDD

LAB/BRANCH
Laboratory of Carcinogen Metabolism

SECTION
Analytical Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.5	PROFESSIONAL: 1.3	OTHER: 1.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The laboratory is involved in carrying out independent research, as well as participating in collaborative projects on carcinogenesis related problems, where mass spectrometry and nuclear magnetic resonance spectroscopy can be used as analytical tools to determine the structure and to confirm the identity of organic molecules, or to monitor chemical or biochemical reaction processes of interest. Studies include: (1) the identification of carcinogen metabolites in activation and metabolism studies; (2) the identification of naturally occurring potential carcinogens in the environment; (3) elucidation of mass spectral fragmentation mechanisms; (4) development of methods for derivatization and analysis of carcinogens; (5) analysis of bioactive materials that may play a role in cancer causation and prevention mechanisms, such as vitamin A derivatives.

Project Description

Objectives: (1) To study in some detail the mass spectra of carcinogens such as N-nitroso compounds, aromatic hydrocarbons and aromatic amines, as well as some of their possible metabolites, with the aim of applying this knowledge to develop appropriate analytical methods. (2) To apply the mass spectrometry and nuclear magnetic resonance spectroscopy (NMR) methods for the analysis and identification of metabolites in carcinogen activation and metabolism studies. (3) To elucidate the chemical structure of certain natural bioactive materials, or of their synthetic analogs, that may play a role in cancer causation and prevention mechanisms. (4) To synthesize appropriate unlabelled and stable isotope labeled standards for spectral and other studies.

Methods Employed: An in-house high resolution double focusing mass spectrometer system was used, interfaced with a gas chromatograph, and run in the electron impact ionization mode. A number of synthetic and metabolic samples were also prepurified by collaborators using high pressure liquid chromatography. In some cases other suitable chemical and spectral characterizations were necessary. The Section's high resolution superconducting-magnet nuclear magnetic resonance (NMR) spectrometer gives important complementary information to mass spectrometry in structural and other studies. The NMR technique is also being applied to the analysis of materials that are thermally unstable or non-volatile, i.e., too polar to be vaporized for mass spectral measurements.

Major Findings: The analytical instrumentation program operates at three levels of effort: first, we engage in independent projects on our own initiative; second, the facility supports the chemical studies related to nitrosamines and metal complexes within the Analytical Chemistry Section; third, the group engages in collaborative studies on projects of mutual interest with investigators within the Laboratory of Carcinogen Metabolism and elsewhere within the Institute.

Independent studies are continuing to examine, in detail, the mass spectral behavior of specific classes of compounds, particularly the aliphatic dialkylnitrosamines, α-acetoxydialkylnitrosamines, and polycyclic aromatic hydrocarbon derivatives, so that the information gained increases the predictive value of the method in structural confirmation of known compounds or in determination of related, yet unknown materials. The current studies utilized N-nitrosomethyl-n-butylamine (NMBA) and five of its deuterated analogs, as well as the unlabeled analogs N-nitrosomethyl-n-hexylamine and N-nitrosomethyl-n-octylamine as substrates for low and high resolution spectral measurements and for metastable defocusing experiments. Results indicate that the nitrosamino moiety exerts a strong directing effect on the electron impact induced fragmentation pathways of these molecules. It was found that the intramolecular hydrogen abstraction by the nitrosamino oxygen atom is nonspecific in NMBA in that the C-2 and C-3 positions of the butyl chain provide the hydrogen to an approximately equal extent, but that hydrogens at other positions are not involved. A more specific hydrogen abstraction is involved in the formation of the M^+-propene ion in the spectrum of NMBA. Labeling studies indicate that the hydrogen retained on the charged fragment originated almost entirely from C-3 of the butyl chain. Double hydrogen rearrangements from the butyl side chain to the nitrosamino moiety, followed by C-N bond cleavage, account for the formation of the protonated diazotic acid ion (M/Z 61, $CH_3N_2OH_2$). The latter ion is characteristic of all 3 homologous N-methylalkylnitrosamines examined. The occurrence of a more fundamental skeletal rearrangement is indicated by ions $C_4H_9O^+$ and $C_4H_8ON^+$ in the spectrum of NMBA. These two species are best derived from rearranged molecular ion(s) which are formed upon n-butyl migration to the nitrosamino moiety, most likely to the oxygen atom. These and other characteristically more simple fragmentations serve to explain the behavior of NMBA in the mass spectrometer, and it is expected that generalizations can be made to explain more fully the behavior of other related N-nitroso compounds.

Biological studies by others indicated that the activated carcinogenic metabolite of benzo(a)pyrene [B(a)P] is the 7,8-*trans*-dihydrodiol-9,10-epoxide derivative. Carbon 10 of this molecule possesses a special chemical reactivity toward nucleophiles, and we have found this reactivity to be exhibited in other 7,8,9,10-tetrahydro-B(a)P derivatives. Our analytical studies necessitated the synthesis of several O-18 and deuterium labeled analogs of 7,8,9,10-tetrahydrobenzo(a)pyrenediols and triols. Preparation of the isopropylidene (acetonide) derivatives of the latter polyhydroxylated compounds gave information on the relative configuration of the various hydroxyl substituents. In addition, derivatization made these compounds amenable to mass spectral characterization and the spectra exhibited distinct differences depending on the location of the acetonide moiety in the molecule. Use of labeled derivatizing reagents also demonstrated that the mechanism of this derivatization is anomalous when a substituent at C-10 of the molecule is involved. These studies are continuing.

In collaborative studies within the Laboratory, *in vivo* metabolic studies of 2,4-diaminoanisole and 2,4-diaminotoluene in the rat are progressing. Recent efforts have been concerned with mass spectral and especially NMR characterization of at least three major urinary water soluble (glucuronide or sulfate) metabolites. Natural abundance ^{13}C NMR spectra were performed including proton noise decoupling and off-resonance decoupling methods for the 2-N-hydroxy derivatives of 4-acetyl-2,4-diaminoanisole and of its toluene analog; aromatic ring hydroxylated derivatives, at carbon 5, of the 2,4-diacetylated title compounds; and the 4-acetylamino-2-glycolamido and the 2-acetylamino-4-glycolamido derivatives of anisole. Full characterization of these molecular types serves to support the structural studies on the metabolites themselves.

Several collaborative projects are concerned with investigations of bioactive materials that may play a role in cancer prevention mechanisms. In joint studies with Dr. Sporn's group, mass spectral studies allowed characterization of all-*trans*-retinyl-β-glucuronide as a biliary metabolite of all-*trans*-retinoic acid. It was found that the dose-dependent clearance of retinoic acid in plasma is not dependent on the enterohepatic recirculation of retinoic acid, whereas the plasma levels of retinoic acid metabolites do depend on the enterohepatic system. In collaborative studies with Dr. DeLuca's group, mass spectral studies contributed key evidence for the chemical characterization of methoxyretrovitamin A_1 methyl ether, the *in vitro* acid hydrolysis product of the biologically active 5,6-epoxyretinyl phosphate. This *in vitro* product is closely related to the retroretinoid that was formed biologically from 5,6-epoxyretinol in spontaneously transformed mouse 3T12 cells. In the antibiotics field, detailed proton NMR measurements, including double resonance experiments, were carried out on FCRC #2064A antibiotic and the structure was confirmed to be identical to the known Gilvocarcin V, active against several experimental tumors. Collaborative studies with Dr. U. Weiss, using high resolution mass spectrometry, identified a closely related promising antibiotic, called Chrysomicin, with the structural studies still continuing.

Significance to Biomedical Research and the Program of the Institute: The understanding of the mass spectral behavior of specific classes of organic compounds is a prerequisite to the interpretation of spectra and to the structural determination of a number of biologically important compounds. Toward this goal, detailed spectral studies on known N-nitroso compounds and polycyclic aromatic compounds have been valuable. Establishing the exact molecular structure and conformation of relevant biomolecules by nuclear magnetic resonance and other techniques is necessary in a modern approach for ultimate understanding of the complex molecular transformations taking place in living systems, particularly in cancer causation mechanisms. Structural studies on metabolites of aromatic amines and of polycyclic aromatic hydrocarbons have given insight into the pathways of carcinogen degradation or activation. Identification of carcinogens or of potential carcinogens in the immediate human environment or in food staples is important in developing prevention methods.

Proposed Course: The studies described above will be completed and published. A number of metabolic studies are continuing and their significance to cancer causation will be evaluated.

Publications:

Börzsönyi, M., Torok, G., Pinter, A., Nadasdi, L. and Roller, P. P.: Carcinogenic effect of dinitrosopiperazine in adult Swiss mice and after transplacental or lactational exposure. Cancer Res. 40: 2925-2927, 1980.

Frolik, C. A., Roller, P. P., Roberts, A. B. and Sporn, M. B.: In vitro and in vivo metabolism of all-trans- and 13-cis-retinoic acid in hamsters. Identification of 13-cis-4-oxoretinoic acid. J. Biol. Chem. 255: 8057-8062, 1980.

Roller, P. P., Keefer, L. K. and Slavin, B. W.: Inhibitory agents and chemical mechanisms in the dihalomethane mediated nitrosation of amines with solid nitrite. In Walker, E. A., Griciute, L., and Börzsönyi, M. (Eds.): N-Nitroso Compounds: Analysis, Formation and Occurrence. IARC Scientific Publication No. 31, International Agency for Research on Cancer, Lyon, 1980, pp. 119-128.

Frolik, C. A., and Roller, P. P.: The role of vitamin A in the prevention of epithelial cancer. In Aszalos, A. (Ed): Antitumor Compounds of Natural Origin. Vol. II., CRC Press, Inc., Boca Raton, Florida, 1981, Chapter 4.

Swanson, B. N., Frolik, C. A., Zaharevitz, D. W., Roller, P. P. and Sporn, M. B.: Dose-dependent kinetics of all-trans-retinoic acid in rats: Plasma levels and excretion into bile, urine and feces. Biochem. Pharmacol. 30: 107-113, 1981.

Roller, P. P., Keefer, L. K., Bradford, W. W., and Reist, E. J.: Synthesis, analysis, and stability studies of ^{14}C-methyl(acetoxymethyl) nitrosamine. J. Labelled Compd. Radiopharm. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04582-06 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Structure Activity Correlations in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lionel A. Poirier	Chief, Nutrition & Metabolism Section	LCM NCI
OTHER:	Camille Hyde	Staff Fellow	LCM NCI
	Lawrence Lanier	Biological Laboratory Technician	LCM NCI

COOPERATING UNITS (if any)
Dr. Martin Wenk, Microbiological Associates, Bethesda, MD

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.7	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The antagonism between the essential divalent metals calcium and magnesium and the divalent metal carcinogens, lead, nickel and cadmium are under investigation in metabolic and in carcinogenecity studies. Magnesium and calcium completely prevented lung adenoma production in strain A mice by nickel and lead acetates. Calcium prevented the inhibition of cadmium of thymidine incorporation into DNA.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The accumulation of evidence has indicated that the activated form of most, if not all, chemical carcinogens consists of a reactive electrophile. Possible mechanisms by which chemicals of diverse structure exert their carcinogenic activity are proposed from a knowledge of their chemical reactivity, structure, metabolic pathways and mutagenic activity.

Methods Employed: The carcinogenic activities of previously untested compounds are tested by standard protocols, generally by the production of mouse lung adenomas in strain A mice. Alternate carcinogenesis studies are done by long-term feeding, injection or intubation of the suspect compound into rats, followed by examination for tumors at necropsy. Thymidine incorporation into the DNA of rat liver cells is measured by standard radioisotopic, cell culture and ultracentrifugation techniques.

Major Findings: The tumorigenic activities of nickel acetate and lead subacetate towards the lungs of strain A mice were completely prevented by the simultaneous administration of calcium acetate and of magnesium acetate. Cadmium chloride inhibited the uptake of thymidine into the DNA of rat liver cells in culture; such inhibition was prevented by increased levels of calcium in the medium.

Significance to Biomedical Research and the Program of the Institute: The aim of these studies is to increase the base of theoretical knowledge by which the potential carcinogenic hazards to man of previously untested carcinogens can be estimated. The evidence accumulated to date indicates that an antagonism to the divalent cations calcium or magnesium may be part of the mechanism by which the divalent metal carcinogens exert their activity.

Proposed Course: The carcinogenicity of inorganic carcinogens will be examined. These include oxidized electrophilic arsenic and chromium compounds. Attempts will be made to extend the observed antagonism between carcinogenic divalent metals and calcium and magnesium.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04589-04 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Endocrine Factors in Tumor Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hajime Nawata	Visiting Associate	LCM NCI
CO-PI:	Lionel A. Poirier	Chief, Nutrition & Metabolism Section	LCM NCI
OTHER:	Richard S. Yamamoto	Chemist	LCM NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of hormones on the induction of ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), and polyamine levels in vivo are being studied. Previous studies have shown that ODC induction may be a marker for tumor promoters. Hormones often increase the biological activities of complete carcinogens. ODC is used to study the degree of biochemical similarity between hormones and promoters. The present studies indicate that estradiol produces renal adenocarcinomas in the hamster and induces renal ODC and SAMDC in both the rat and hamster. The induction of renal ODC in the rat by estrogens is proportional to their binding in vitro to cytoplasmic estradiol binding sites. ODC levels in the ovary and uterus of female rats are inversely correlated with each other.

Project Description

Objectives: The major objective of these studies is to determine whether the enhancement of the activity of certain complete carcinogens by hormones is due to a promoting activity of the hormone.

Previous studies have demonstrated that certain hormones often increase the activity of specific carcinogens towards their target organs. In theory, such effects may be due to (a) altered metabolism, increasing the effective intracellular concentration of ultimate carcinogens, (b) promotion of initiated cells and (c) other incompletely defined co-carcinogenic effects. Previous studies have shown that hormones frequently alter carcinogen metabolism. We are attempting to show that hormones may act as promoters. The induction of ornithine decarboxylase (ODC) is used as a marker of promoting-like activity.

Methods Employed: The enzymes ODC and S-adenosylmethionine decarboxylase are assayed by trapping CO_2. Tissue levels of polyamines are determined by thin-layer chromatography and fluorimetric analysis. The enzymes and the polyamines are determined in several organs of intact and hormonally-ablated rats and hamsters, which have been treated with hormones.

Major Findings: (a) Testosterone enhances and estradiol inhibits the induction of ODC by growth hormone in the livers of rats. (b) Renal ODC, SAMDC and polyamine levels are increased by estradiol administration. The induction of renal ODC in the rat by estrogen analogues is proportional to their *in vitro* interaction with cytoplasmic estradiol binding sites. (c) There is an inverse correlation between the levels of ODC and SAMDC in the ovary of rats and their corresponding level in the uterus. The observed effects of exogenous hormones on the tissue levels of ODC generally parallel the effects of the same hormones on carcinogenesis.

Significance to Biomedical Research and the Program of the Institute: A major goal of the NCI is to determine the mechanism of action of chemical carcinogens. These studies attempt to clarify the contribution of hormones to the carcinogenic process in man.

Proposed Course: These studies have been completed and the project is now terminated.

Publications:

Nawata, H., Yamamoto, R. S. and Poirier, L. A.: An inverse correlation between uterine and ovarian levels of ornithine decarboxylase and S-adenosylmethionine decarboxylase in the rat. *Proc. Soc. Exp. Biol. Med.* (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04617-16 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Carcinogen Screening Operations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elizabeth K. Weisburger	Chief	LCM NCI
OTHER:	Timothy Benjamin	Chemist	LCM NCI
	Ritva P. Evarts	Veterinary Medical Officer	LCM NCI

COOPERATING UNITS (if any)
Dr. M. B. Shimkin, University of California, La Jolla, CA; Dr. B. Ulland, Hazleton Labs., VA; Dr. Spangler, Microbiological Associates, Bethesda, MD; and Dr. A. Krishna Murthy, Mason Research Institute, Worcester, MA.

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The chronic effects of environmental chemicals or of mixtures of chemicals are determined in suitable animal models. Factors influencing the outcome, such as sex, strain and species of animal, are investigated.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Responsibilities are assumed on the design, performance, and evaluation of studies in either contract or intramural laboratories, on the chronic effect and carcinogenicity of chemicals or of mixtures of chemicals. The aim of these investigations is to gather information on hazards involved in handling certain chemicals or drugs, and also on the mechanisms underlying joint effects in mixtures. Of particular interest are such mixtures which tend to give increased or decreased overall carcinogenic effect. Of concern also is the methodology of carcinogen screening, with emphasis on improvements in sensitivity, speed and economy. Short-term assays are under development, taking into account the need to secure biochemical activation of most environmental agents. Guidelines for assessment of the carcinogenic potential of chemicals are developed, by consideration of their chemical structure, metabolic pathways and mutagenic activity.

Methods Employed: Attempts are made to secure information on chemicals representing the greatest hazard to the largest number of people. Chemicals and drugs are rated as to priority on this basis, as well as other criteria, such as relationship to known carcinogens, epidemiologic observations, indications of specific toxicity and related factors. Standard protocols, as well as newly designed protocols, attempting to increase the sensitivity and speed of such tests are utilized. The carcinogenic and mutagenic activities of various compounds are determined in appropriate systems, either directly or after metabolic activation. Exploratory meetings with relevant national and international organizations in industry, in government and in university environments are setting the stage for a concerted development effort in this area.

Major Findings: Industrial or environmental chemicals. Papers are in press or in preparation on the long-term effects of various industrial or environmental chemicals such as pesticides, industrial intermediates and dyestuff intermediates.

Significance to Biomedical Research and the Program of the Institute: Many substances previously used industrially or in the environment had not yet been evaluated for chronic toxicity and possible carcinogenicity. Until the Toxic Substances Control Act is fully in effect, it is important to secure information on these effects. Cancer in man has been observed previously as a result of exposure to chemicals often demonstrated to be carcinogenic. The aim of this program is to prevent unintentional exposure of man which results in future neoplastic disease.

Proposed Course: It is anticipated that due to the reorganization of the group this project will be phased out.

Publications:

Fleischman, R. W., Baker, J. R., Hagopian, M., Wade, G. G., Hayden, D. W., Smith, E. R., Weisburger, J. H. and Weisburger, E. K.: Carcinogenesis bioassay of acetamide, hexanamide, adipamide, urea and p-tolylurea in mice and rats. J. Environ. Pathol. Toxicol. 3: 149-170, 1980.

Weisburger, E. K., Krishna Murthy, A. S., Fleischman, R. W. and Hagopian, M.: Carcinogenicity of 4-chloro-o-phenylenediamine, 4-chloro-m-phenylenediamine, and 2-chloro-p-phenylenediamine in Fischer 344 rats and $6C3F_1$ mice. *Carcinogenesis* 1: 495-499, 1980.

Benjamin, T., Evarts, R. P., Reddy, T. V. and Weisburger, E. K.: The effect of 2,2'-diaminodiphenyldisulfide, a resin hardener, in rats. *J. Toxicol. Environ. Health* 7: 69-81, 1981.

Marzulli, F. N. and Weisburger, E. K.: Cosmetics. In Sontag, J. M. (Ed.): *Carcinogens in Industry and the Environment*. New York, Marcel Dekker, 1981, pp. 573-582.

Weisburger, E. K.: Chemical carcinogenesis and its relevance for the general population. In Sax, N. Irving (Ed.): *Cancer Causing Chemicals*. New York, Van Nostrand Reinhold, 1981, pp. 3-13.

Weisburger, E. K.: Carcinogenic agents. In Pradhan, S. N. (Ed.): *Textbook of Pharmacology*. St. Louis, Missouri, C. V. Mosby Co. (in press).

Weisburger, E. K.: Carcinogenicity tests on pesticides. American Chemical Society, Atlanta, Georgia, Raven or Academic Press (in press).

Weisburger, E. K.: Species differences in response to aromatic amines. In: Proceedings of Symposium on Organ and Species Specificity in Chemical Carcinogenesis, Raleigh, NC, March 1981 (in press).

Weisburger, E. K., Ulland, B. M., Nam, J. M., Gart, J. J. and Weisburger, J. H.: Carcinogenicity tests of certain environmental and industrial chemicals. *J. Natl. Cancer Inst.* (in press).

Weisburger, E. K.: Halogenated substances: Environmental and industrial materials. In Khan, M. A. Q. (Ed.): *Halogenated Hydrocarbons: Health and Ecological Effects*. New York, Pergamon Press (in press).

Weisburger, E. K.: Techniques for carcinogenicity studies. *Cancer Res.* (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04618-16 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Endogenous and Exogenous Factors in Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ritva P. Evarts	Veterinary Medical Officer	LCM NCI
OTHER:	Carolyn B. Ligon	Microbiologist	LCM NCI
	Elizabeth K. Weisburger	Chief	LCM NCI

COOPERATING UNITS (if any)
Dr. B. Cockrell, Experimental Pathology Laboratories; Developmental Pharmacology Branch, NICHHD

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects structural alterations may have on the carcinogenicity or toxicity of various compounds are determined. Methods to inhibit the action of known carcinogens by administration of other compounds are devised. The biochemical, morphological, and physiological bases for such inhibitory effects are studied. Characterization and isolation of cytochrome P-450 species involved in the metabolism of dimethylnitrosamine (DMN) are under investigation. The importance of the active liver microsomal enzyme system on the tumor production in the peripheral tissues is evaluated.

Project Description

Objectives: Investigations to determine the intimate factors involved in initiation and development of neoplasia are performed. The current aims are 1) to isolate and characterize the microsomal cytochrome P-450 species that are involved in the metabolism of DMN; 2) to study the effect of the closely related chemicals 2,4-diaminoanisole and m-phenylenediamine, on the morphology of the thyroid gland and on thyroid tumor production, and 3) to determine the function of liver microsomal enzyme systems in carcinogenesis of peripheral tissue, especially of kidney and bladder.

Methods Employed: Evaluation of the effects of the chemical factors in liver microsomal enzyme systems are based on *in vivo* and *in vitro* studies of different components of the liver mixed-function oxidase system. Isolation and characterization of the different components of this system have been accomplished using different column chromatographic techniques and slab gel electrophoresis. The Ames *Salmonella* system has been used for evaluation of the effect of the chemical treatment on the metabolism of DMN.

Major Findings: (1) Pyrazole. Several pyrazole derivatives are clinically important because of their antipyretic, analgesic and anti-inflammatory properties. Pyrazole was a potent inducer of the activity of low-substrate level dimethylnitrosamine demethylase (DMN-d), but had an opposite effect on arylhydrocarbon hydroxylase. Pyrazole-induced DMN-d activity was NADPH-dependent and was inhibited by CO. Cytochrome P-450 was slightly increased by pyrazole and its CO-complex gave an absorption maximum around 451 nm. When the microsomal proteins were separated using SDS polyacrylamide gel electrophoresis, a large increase in the electrophoretic band at about 52,000 molecular weight was found in the liver microsomes of pyrazole-treated animals. In the *Salmonella* test system a ten-fold increase in revertants was found when microsomes from pyrazole-injected animals were used to activate dimethylnitrosamine, whereas no such increase was seen with microsomes from phenobarbital- and methylcholanthrene-injected animals.

(2) 2,4-Diaminoanisole (2,4-DAA). Feeding the carcinogenic hair dye component 2,4-DAA to Fischer female rats for four weeks revealed a goitrogenic effect, with prominent pigmentation of the epithelium of the thyroid follicular cells. When the noncarcinogenic analog m-phenylenediamine (m-PDA) was fed simultaneously with 2,4-DAA, no pigmentation of the thyroid epithelium was observed.

The relevance of this difference to tumor production by 2,4-DAA is under investigation. A long-term experiment was initiated to determine whether the goitrogenic effect alone is necessary for the production of the thyroid tumors or if the accumulation of the dark brown pigment in the thyroid epithelium is a necessary step towards the production of thyroid neoplasms. If the pigmentation of thyroid epithelium is involved in the process of carcinogenesis, simultaneous application of m-PDA might inhibit the carcinogenic effect of 2,4-DAA.

(3) Partial hepatectomy lowers the activity of dimethylnitrosamine metabolizing enzymes in the liver, but activity returns to the normal levels after a few days. In general, the activity of drug-metabolizing enzymes, which also activate chemical carcinogens to alkylating species, is highest in liver, whereas peripheral

tissues are often targets for tumor formation. Our goal was to study the importance of an active liver mixed-function oxidase system in carcinogenesis. We took advantage of the depressing effect of partial hepatectomy on liver DMN-d to study the occurrence of kidney tumors as a result of DMN administration at different time intervals after partial hepatectomy. There was an inverse relationship between the occurrence of kidney tumors and the activity of liver DMN-d. Besides affording an insight into the mechanism of DMN activation, the system may be a useful model for study of kidney tumors.

Significance to Biomedical Research and the Program of the Institute: The specific chemical and dietary factors affecting the metabolism of DMN are largely unknown. The effect drugs or chemicals containing the pyrazole nucleus have on the toxicity and carcinogenicity of DMN will be elucidated.

Proposed Course: Emphasis will continue on the function of the liver mixed-function oxidase system in the metabolism of DMN. Reconstituted microsomal enzyme systems will be used to determine the specific cytochrome P-450 species involved in the metabolism of DMN. This requires isolation of cytochrome C-reductase and cytochrome P-450.

Publications:

Evarts, R. P. and Brown, C. A.: 2,4-Diaminoanisole: Early effect on thyroid gland morphology and late effect on glandular tissues of Fischer 344 rats. J. Natl. Cancer Inst. 65: 197-204, 1980.

Gothoskar, S. V. and Weisburger, E. K.: Protection by testosterone propionate against the nephrotoxicity of 2-aminoanthraquinone in Fischer rats. Med. Biol. 58: 281-284, 1980.

Evarts, R. P. and Mostafa, M. H.: Effects of indole and tryptophan on cytochrome P-450, dimethylnitrosamine demethylase and arylhydrocarbon hydroxylase activities. Biochem. Pharmac. 30: 517-522, 1981.

Evarts, R. P. and Brown, C. A.: 2,4-Diaminoanisole-induced thyroid pigmentation in rats inhibited by m-phenylenediamine. Toxicol. Letters (in press).

Mostafa, M. H., Ruchirawat, M. and Weisburger, E. K.: Comparative studies on the effects of various microsomal enzyme inducers on the N-demethylation of dimethylnitrosamine. Biochem. Pharmac. (in press).

Mostafa, M. H., Ruchirawat, M. and Weisburger, E. K.: Effect of indole on dimethylnitrosamine-demethylase in rats treated with carbon tetrachloride. Fd Cosmet. Toxicol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04619-16 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Metabolism of Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Preston H. Grantham	Chemist	LCM NCI
OTHER:	Timothy Benjamin	Chemist	LCM NCI
	R. Ramanathan	Visiting Associate	LCM NCI
	Peter P. Roller	Chemist	LCM NCI
	Elizabeth K. Weisburger	Chief	LCM NCI

COOPERATING UNITS (if any)
Analytical Chemistry Section, LCM, NCI

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolic pathways of chemical carcinogens, generally various aromatic amines, are studied both in animals or in isolated tissues. Metabolites are separated by various techniques such as thin-layer, column, gas-liquid or high pressure liquid chromatography. Identification is made through physico-chemical means such as mass spectrometry, nuclear magnetic resonance and ultraviolet spectra. Interaction of metabolites with cellular constituents is determined. The effect inhibitors or promoters of the carcinogens exhibit on the metabolic patterns is studied.

Project Description

Objectives: The aim of this research is to gather data relevant to the etiology of neoplasia at the molecular level. To this end chemical carcinogens, especially those of the aromatic amine type, are being utilized. Their metabolism and interaction with host tissues and specific targets are studied. Simplified, yet realistic, model systems are devised in order to gain an understanding of the fundamental events operating in chemical carcinogenesis. Specific inhibitors and accelerators of the carcinogenic process are applied as tools to develop information on and permit discrimination between biochemical events directly involved in the neoplastic change and those representing other reactions.

Methods Employed: Biochemical and pharmacological techniques are applied to determine the metabolism of 2,4-diaminoanisole, N-hydroxy-N-2-fluorenylacetamide, and related compounds in various animal species. This includes study of the enzyme systems concerned with certain metabolic steps, development and application of procedures for the separation, purification and analysis of macromolecular constituents such as DNA, RNA, and proteins from tissues of animals treated with chemical carcinogens and from control animals. The metabolism of chemical carcinogens and related compounds, and interaction with host targets are examined in vivo and in vitro in the presence of various chemicals or antibiotics which affect the carcinogenic process.

Major Findings: (1) Metabolism of 2,4-Diaminoanisole (2,4-DAA) in Hamsters. The metabolic study of 2,4-DAA was continued in order to identify the metabolite or metabolites which yield 2-acetamido-5-methoxy-1,4-benzoquinone during workup of the urine. Major bands were isolated from the glucuronide fraction of hamster urine using the lead acetate method. These bands were then separated by additional chromatography. One band was conclusively identified by NMR and mass spectra as the glucuronide of 2,4-diacetylaminophenol. Another band was identified as the glucuronide of 5-hydroxy-4-acetylamino-2-aminoanisole. This metabolite would explain the finding of the benzoquinone during workup of the urine.

(2) Water soluble metabolites of 2,4-DAA in the rat. Saturation of the aqueous phase with ammonium sulfate showed that further materials were extractable from this phase with ether-ethanol, followed by chromatography and identification by NMR and mass spectral techniques. A possible metabolite which was identified, with molecular ion m/e 270, supports a structure of 3,8-diamino-2,9-dimethoxybenzo[c]cinnoline, indicating the probability of an N-hydroxylaminoanisole derivative as an intermediate. Another compound isolated, with m/e of 196, very likely is 2-acetylamino-5-hydroxy-p-anisidine. Acetylation of this metabolite yielded a compound with m/e of 238 with the same retention time on HPLC and TLC as the synthetic reference compound 5-hydroxy-2,4-diacetylaminoanisole. A third compound with m/e of 166 and/or 196 may be 2-amino-4-acetylaminophenol (m/e 166), and 2-(glycolamido)-p-anisidine or an isomer (m/e 196). The tentative identification of these metabolites indicates that a significant fraction of the water soluble metabolites may be aminohydroxy compounds which are not extractable by our extraction system.

(3) Fraction of urinary metabolites of 2,4-DAA by column extraction techniques. Sep Pak and extraction columns, relatively new developments, were employed. Using these columns, as compared with our usual extraction techniques to work

up urine, showed that more of the urinary metabolites could be isolated in a form amenable for identification by the column extraction method. Similarly, with urine from hamsters fed 2,4-DAA, the column-extraction method led to obtaining more material in fractions amenable to our usual workup procedures. This new technique may thus facilitate the identification of the water soluble metabolites of 2,4-DAA.

(4) Metabolites of 2,4-DAA after partial hepatectomy of rats. Preliminary studies have shown that partial hepatectomy decreases the rate of metabolism of 2,4-DAA so that more of a dose is excreted at later time periods. However, at the end of 48 hours, the fraction of dose excreted is very similar to that excreted by controls. Likewise, after subcutaneous administration of 2,4-DAA to rats, the metabolic pattern was very similar to that shown after intraperitoneal administration.

(5) Metabolism of 2,4-DAA in female rats. Our previous studies have generally been done with male rats. A study on females showed that the metabolic pattern and extraction pattern of metabolites in the urine was very similar to that of the male rats.

(6) Mutagenicity of urinary metabolites of 2,4-DAA. Urines from animals given 2,4-DAA for varying time periods were separated into the various fractions which were assayed by the Ames test system. All fractions, including the free, glucuronide and sulfate conjugates, showed some mutagenic activity. The extracts are also incubated with S-9 mixtures to determine whether activation was possible. These studies are continuing.

In addition, the major metabolites of 2,4-DAA such as 4-acetylamino-2-aminoanisole and 2,4-diacetylaminoanisole, as well as the compound 2-acetylamino-4-aminoanisole (which is not a metabolite) were administered to rats. The urines were collected and fractions separated and examined using the Ames test system. These experiments will focus on the mechanisms involved in the activation of 2,4-DAA.

Significance to Biomedical Research and the Program of the Institute: By the utilization of certain chemical carcinogens, the molecular mechanism of steps leading to cancer is being explored. If the entire process is viewed as a series of steps from the primary interaction between an agent and cellular constituents, followed by multiplication of abnormal cells which, in turn, can undergo further transformations, there are a number of points where one could prevent or even reverse such interactions. Thus, the ultimate aim of these studies is to comprehend fully the sequence of complex reactions leading to cancer and eventually to be in a position to prevent them under realistic conditions in man.

Proposed Course: The program of elucidating the biochemistry of carcinogenesis, our eventual goal, is a continuing, long-term effort. Certain promising leads which have been discussed in this account will be followed up in further experiments.

Publications:

Gothoskar, S. V., Chitnis, M. P. and Weisburger, E. K.: Proteinuria in the Fischer rat by feeding 2-aminoanthraquinone. Med. Biol. 58: 337-340, 1980.

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Weisburger, E. K.: Laboratory chemicals: N-2-Fluorenylacetamide and derivatives. In Sontag, J. M. (Ed.): Carcinogens in Industry and the Environment. New York, Marcel Dekker, 1981, pp. 583-666.

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Ramanathan, R., Reddy, T. V. and Weisburger, E. K.: Alterations in drug metabolizing enzymes during feeding of the carcinogen 2-aminoanthraquinone. Toxicol. Appl. Pharmac. (in press).

Ruchirawat, M., Grantham, P. H., Benjamin, T. and Weisburger, E. K.: Effect of phenobarbital pretreatment on the toxicity and metabolism of 2,4-diaminoanisole. Biochem. Pharmac. (in press).

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Weisburger, E. K. and Reddy, T. V.: Chemists and carcinogens -- Exogenous and endogenous. In: Solution Behavior of Surfactants -- Theoretical and Applied Aspects, New York, Plenum Press (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04620-16 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mode of Action of Chemical Carcinogens -- Chemical Investigations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Timothy Benjamin	Chemist	LCM NCI
OTHER:	Robert C. Atkins	Guest Worker	LCM NCI
	Elizabeth K. Weisburger	Chief	LCM NCI

COOPERATING UNITS (if any)
Analytical Chemistry Section, LCM, NCI; James Madison University, Harrisonburg, VA

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project serves as a supporting function to the studies on metabolism of various toxic or carcinogenic agents in this Laboratory. The purity of compounds being investigated for toxicity or carcinogenicity is determined along with pertinent physical characteristics, useful in the design of the experiments. Possible metabolites of various aromatic amines, such as 2,4-toluenediamine, 2,4-diaminoanisole, and 2-aminoanthraquinone are synthesized as reference materials for metabolism studies. Methods for separation of aromatic amines and their metabolites are investigated.

Project Description

Objectives: Syntheses of possible metabolites of the carcinogens 2,4-diaminoanisole and 2,4-toluenediamine were conducted to aid in the identification and analysis of substances isolated in the metabolism studies. An additional benefit was the acquisition of spectroscopic data (primarily nuclear magnetic resonance) which would aid in the characterization of other metabolites.

Methods Employed: Standard laboratory synthetic methods and reactions were utilized to convert commercially available starting materials into the chemical substances of interest. Reaction products were fully characterized. Techniques utilized included thin layer and high pressure liquid chromatography, infrared spectrometry, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry. Chemical substances unreported in the literature were analyzed by high resolution mass spectrometry (Analytical Chemistry Section). Most proton NMR spectra and all carbon-13 NMR spectra were obtained on the high field instrument present in the Analytical Chemistry Section.

Major Findings: (1) Hydroxylamine syntheses. The syntheses of two hydroxylamine derivatives, namely 4-acetylamino-2-hydroxylaminoanisole and 4-acetylamino-2-hydroxylaminotoluene, were completed by zinc reduction of the corresponding nitro derivatives. The substances were obtained as mixtures, and purification was difficult due to the instability of the N-hydroxy derivatives in solution.

(2) Ring hydroxy syntheses. The syntheses of two ring hydroxylated metabolites, 2,4-diacetylamino-5-hydroxyanisole and 2,4-diacetylamino-5-hydroxytoluene, were accomplished through nitration, catalytic reduction in acetic anhydride to yield a triacetyl derivative, and final deacetylation with a metal-hydride reagent.

(3) Glycolamide synthesis. 4-Acetylamino-2-glycolamidoanisole and 2-acetylamino-4-glycolamidoanisole were obtained by reaction of the corresponding amines with glycolic (hydroxyacetic) acid.

(4) Nuclear magnetic resonance spectroscopy. The metabolites mentioned above, plus several key synthetic intermediates, were analyzed in detail by high field NMR spectroscoy. Data were obtained regarding the natural abundance carbon-13 spectra of these substances. Both proton noise decoupled and off-resonance decoupled spectra were obtained. Analysis of these spectra has resulted in assignment of chemical shifts of the carbon atoms in the synthetic metabolites. Consideration of peak intensities allowed distinction to be made between substituted and unsubstituted carbon atoms on the skeleton; the data will be helpful in the characterization of unknown metabolites. The Nuclear Overhauser Enhancement effect was also observed in the proton spectra of the synthetic metabolites. These results allow the determination of the spatial relationship of neighboring substituents.

NMR spectra, using time-averaging Fourier Transform techniques, were obtained on several samples of metabolites of unknown structure isolated during animal studies. Deuterium exchange experiments were also carried out in order to identify acidic hydrogens present in these substances.

Significance to Biomedical Research and the Program of the Institute: These studies aid in the identification and characterization of carcinogen metabolites, and therefore serve as an important adjunct to the broad programs on the mechanism of action of chemical carcinogens pursued in this laboratory.

Proposed Course: The synthesis and spectroscopic identification and analysis of metabolites of chemical carcinogens will continue.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04680-11 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Development and Application of In Vitro Systems Involving Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mary J. Wilson	Chemist	LCM NCI
OTHER:	Juanita Brown	Biological Laboratory Technician	LCM NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Epithelial-like cells derived from livers of infant Fischer rats are maintained in culture, either continuous or after frozen storage. Transformation of the cells to a malignant form by known chemical carcinogens and metabolites of known carcinogens is being studied.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Successful culture of epithelial-like cells derived from livers of 8 to 10 day old, Fischer strain 344 rats and malignant transformation of such cultured cells by a variety of chemical carcinogens were discussed in previous reports. Since neoplasia in man and animals involves mainly epithelial cells, our principal aim in current investigations has been to determine further the potential of in vitro epithelial systems for use in rapid bioassay procedures for chemical carcinogens and in examining the biochemical mechanisms of carcinogenesis.

Two problems encountered in past investigations were the relatively long time required to develop cultures that were proliferating adequately to provide enough cells for biochemical investigations and the fact that some sublines of control cells underwent spontaneous malignant transformation as demonstrated by tumor formation following injection of cells into syngeneic hosts. Unfortunately, no distinctive morphological changes were apparent in the cultured cells that induced tumors. In addition, the spontaneous transformation was not correlated with a specific time in culture nor with the number of times a line had undergone subculturing. Therefore, we have emphasized the development of methods for producing a greater number of cultures with similar characteristics so that studies can be repeated as required in more replicable systems. Investigation of pertinent biochemical properties of untreated or chemically exposed cultured cells in respect to the effects of various cultural conditions and in comparison to the characteristics of liver tissue from whole animals should help assess the value of this in vitro system.

Methods Employed: Methods for selecting rat liver cells for culture continue to be developed in this laboratory. This involved detaching and transferring islands of epithelial cells, both mechanically and by trypsinization from cloning cylinders, so that many homogeneous sublines can be cultured from one primary culture. Previous studies in this laboratory have shown that frozen storage of cells did not alter enzyme levels or cause transformation.

Major Findings: Treatment of rat liver cells with ethionine. Irreversible morphological alterations were produced in cultures of liver cells treated for three months with 5.0 mM or 7.5 mM DL-ethionine. Growth in soft agar was observed in both treatment groups after 5 additional months in culture. Only those cells treated with 7.5 mM DL-ethionine produced tumors when injected subcutaneously into syngeneic hosts.

Cells treated with L-S-adenosylethionine (0.2 mM) and L-ethionine (0.2 mM and 0.375 mM), in medium in which methionine was replaced by homocysteine, were able to grow in soft agar after 3 months of treatment and an additional 3 months in culture.

Significance to Biomedical Research and the Program of the Institute: An assay system which can be designed to give results more rapidly, specifically, and reliably than the customary long-term tests for carcinogenicity is highly desirable with the dual purpose of studying fundamental phenomena and the detection of harmful agents in our environment. Full exploitation of workable in vitro systems has not been achieved. It is our goal to develop such systems; an additional advantage of the in vitro systems is that, eventually, they can be established utilizing cells from man.

Hence, carcinogenesis in vitro with human cells avoids the question of species difference, so often raised in evaluating the significance of carcinogen bioassay systems.

Proposed Course: The epithelial cells will continue to be examined for suitability as a carcinogen screening system. Studies in progress with the carcinogen ethionine will be pursued.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05203-01 LCM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mutagenicity of Carcinogens and Their Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard S. Yamamoto	Chemist	LCM NCI
CO-PI:	Nabil M. El-Torkey	Visiting Fellow	LCM NCI
OTHER:	Preston H. Grantham	Chemist	LCM NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.2	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mutagenicity assays using the Salmonella typhimurium mutant strains were initiated. The metabolites of various carcinogens, e.g., N-2-fluorenylacetamide (FAA), 2,4-diaminoanisole (DAA), 1,2-dimethylhydrazine (DMH), N-nitrosomethylurea (NMU), were obtained by administering them to rats and collecting the rat urines, then testing for mutagenicity with the Ames strains. When carcinogen inhibitors were administered with the carcinogens to rats, e.g., acetanilide against FAA, the mutagenicity of the urine increased; non-antagonists did not increase the mutagenic activity. Feeding tyrosine was found to increase the mutagenicity of urine excreted by DAA-treated rats.

PHS-6040
(Rev. 2-81)

Z01 CP 05203-01 LCM

Project Description

Objectives: To test the various metabolites of carcinogens and related non-carcinogens for mutagenic activity; to follow the active mutagen; to study the pathway of the metabolites; and to determine whether other chemicals prevent or enhance the activity of a carcinogen by examining the urine for mutagenic activity.

Methods Employed: The carcinogen with and without antagonists or enhancers is administered to rats. Urine is collected and analyzed for mutagenic activity with the addition of -glucuronidase and/or S-9 (an Araclor-induced activated liver fraction). Urine is also extracted before and after treatment with -glucuronidase followed by sulfatase, then tested for mutagenicity. Usually sufficient activity is found in individual rat urines to allow quantitation and comparison within and between groups.

Major Findings: Urines from animals administered both tyrosine and DAA had greater mutagenic activity with S-9 and -glucuronidase treatment than without, showing that the rat metabolized less DAA in the presence of tyrosine. Similar results were obtained when FAA was administered to rats fed a diet containing acetanilide, a known inhibitor for the carcinogenicity of FAA. DAA responded variably in the presence of acetanilide. In urines from both DAA- and FAA-treated rats, the presence of S-9 and -glucuronidase increased the mutagenic activity several-fold over that of S-9 alone, with no difference in activity with or without -glucuronidase, thus demonstrating that no mutagenic metabolite of FAA or DAA is present in the urine *per se*. One possibility is that the mutagen is activated with S-9 in the proximity of the target and also must be deconjugated before activation. The proportion of metabolites increases following continuous administration of carcinogen; likewise, there also is an increase in the proportion of mutagenic activity in urine following the continuous administration of carcinogen.

Significance to Biomedical Research and the Program of the Institute: The study of mutagenic activity of metabolites is important since most carcinogens are mutagens. If a chemical can be metabolized into a mutagen, the possibility is great that the compound may be carcinogenic. By studying the mutagenicity of metabolites in the urine, one can easily test whether an inhibitor or a promoter is effective against certain carcinogens. Urine of test animals can be readily examined for mutagenicity at various time periods; thus, the progression of effect can be observed.

Proposed Course: Continued studies will be made with this technique to test inhibitors and enhancers, promoters and co-carcinogens, partially metabolized, conjugated or blocked compounds. Following the studies of urines of carcinogen-treated rats, other body tissues, such as blood, plasma, liver, kidney, etc., will be examined for active mutagens.

Publications:

None

CONTRACT NARRATIVE
LABORATORY OF CARCINOGEN METABOLISM
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1981

LITTON-BIONETICS, INC. (N01-CP-01039)

Title: Holding Facility for Small Laboratory Animals

Contractor's Project Director: Dr. Allen Manus

Project Officer (NCI): Dr. Lionel A. Poirier

Objectives: The purpose of the contract is to provide animal holding facilities for rats, mice and hamsters treated with a vareity of chemical carcinogens and fed several different diets and support services for research conducted by the Laboratory of Carcinogen Metabolism.

Methods Employed and Performance: Standard carcinogenesis feeding and injection studies are conducted. Initiation and promotion studies in rat liver are done by administering short pulses of the hepatocarcinogens 2-acetylaminofluorene and N-nitrosodiethylamine. Promotion is performed by feeding methionine antagonists. Typically, carcinogenesis studies are performed for 1.5 to 2 years. Preliminary toxicity studies are conducted to determine the appropriate carcinogen levels and dietary regimens to be employed. Other services include the routine weighing, termination, necropsies, and histological slide preparation from selected tissues of carcinogen-treated animals. Since this is a newly awarded contract, none of the carcinogenesis studies have advanced to an experimental stage where significant results may be expected.

Significance to Biomedical Research and Proposed Course: The prevention and cure of neoplasia hinges on an understanding of the intimate factors involved in the pathogenesis of cancer. Studies undertaken under this contract are expected to provide the essential biological results testing the working hypotheses of the Nutrition and Metabolism Section on the role of methyl donors and metals in carcinogenesis. The animal holding facilities will continue to be used during the administration of chemical carcinogens. The studies will be slightly expanded to include other inorganic carcinogens whose mode of activity is being examined.

Date Contract Initiated: 9/25/80

Current Annual Level: $201,512

SUMMARY REPORT

LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY

October 1, 1980 through September 30, 1981

The major goals of the Laboratory of Cellular and Molecular Biology (LCMB) are to determine the etiology of naturally occurring cancers, to elucidate mechanisms of transformation by carcinogenic agents and to develop experimental strategies capable of prevention of spontaneous and virus-induced tumors. The ultimate aim of these investigations is to apply approaches successful in animal model systems to the identification of causative agents of human malignancies and to the prevention of cancer in man. The primary emphasis of many ongoing investigations within the Laboratory concerns RNA tumor viruses. These viruses are unique among animal viruses in their mode of transmission and the intimate association that has evolved between these agents and cells of a large number and wide variety of vertebrate species. The etiologic role of this virus group has been established for naturally occurring cancers of many species, including some subhuman primates. Certain members of this virus group, so-called "replication-defective" transforming viruses, appear to have arisen by a mechanism involving recombination with cellular transforming genes. As such, these viruses offer an unparalleled opportunity to elucidate the processes by which such genes cause malignancies. Thus, research within the LCMB encompasses efforts to understand the processes involved in malignancy utilizing RNA tumor viruses as models. At the same time, studies are in progress to develop and apply the most sensitive and specific methods of tumor virus detection to the search for related oncogenic viruses of man. Major research efforts of the LCMB in these areas are centered within the Molecular Biology and Experimental Oncology Sections.

During the past year, significant progress has been made by LCMB scientists in the investigation of replication-defective mammalian transforming retroviruses. These viruses have been isolated from a number of mammalian species, including subhuman primates. Our studies have led to the biological characterization of most of the known isolates of this virus group. In each case, these viruses have been shown to possess the capacity to transform cells, but to be replication-defective, requiring a type C RNA virus as a helper. During the past year, we have continued to apply recombinant DNA and nucleotide sequencing techniques to the elucidation of the structure and functions of these viruses and their cell-derived transforming genes.

Abelson murine leukemia virus (A-MuLV) is a mouse transforming retrovirus, which induced lymphosarcomas *in vivo* and transforming fibroblasts, as well as B-lymphoid cells, in tissue *culture*. The integrated proviral genome of A-MuLV was molecularly cloned in *E. coli* using bacteriophage vector λgtWes•λB. A 7.8 kbp DNA fragment *containing* the integrated viral genome was obtained following Eco RI digestion of high molecular weight DNA of A-MuLV nonproducer cells and enrichment by RPC-5 chromatography and preparative gel electrophoresis. Recombinant DNA clones containing a 7.8 kbp Eco RI fragment were shown to contain the entire integrated A-MuLV genome with 5' and 3' ends flanked by 1.8 kbp and 0.2 kbp mink cellular DNA sequences, respectively. A physical map of the proviral genome was generated and revealed that restriction enzymes

Hind III, Bam HI, Cla I, Sal I, Pvu I and Bst III each cleaved at a unique site in the viral genome.

Cloned A-MuLV DNA was shown to transform NIH/3T3 cells at reasonably high efficiency. Moreover, such transformants contained the rescuable A-MuLV genome. In order to localize the region of A-MuLV required for transformation, we measured the infectivity of the proviral genome following exposure to different restriction enzymes. A-MuLV DNA cleaved with Sac I, Bgl I, Pvu I, Bgl II and Pst I lost detectable biologic activity. In contrast, cleavage of A-MuLV proviral DNA with Hind III, Bam HI, or Cla I did not impair transforming activity. To eliminate the possibility that transfecting activity was due to incomplete restriction, A-MuLV subgenomic DNA fragments were prepared and cloned in plasmid vector pBR322. Transfection with subgenomic A-MuLV DNA clones helped to further localize the region of the viral genome necessary for transformation. Efforts are currently under way to clone and compare the normal cellular counterparts of the A-MuLV "leuk" gene in mouse and human cells.

Simian sarcoma virus is the only known transforming retrovirus isolate from a primate. This virus has been very difficult to characterize biochemically due to the presence of a high excess of helper virus in SSV stocks. The integrated form of simian sarcoma virus (SSV) was molecularly cloned in the Charon 16A strain of bacteriophage λ. By transfection analysis, the recombinant viral DNAs demonstrated the ability to transform cells in tissue culture at high efficiency. Such transformants possessed typical SSV morphology, expressed simian sarcoma associated virus (SSAV) gag gene products in the absence of virus release, and released SSV following superinfection with a type C helper virus. A physical map of the 5.8 kbp recombinant viral DNA clone, deduced from restriction endonuclease analysis, revealed a 5.1 kbp SSV genome containing 0.55 kbp long terminal repeats (LTRs) flanked by 0.45 and 0.25 kbp of contiguous host cell sequences. By R-loop analysis, the viral DNA molecule contained two regions of homology to SSAV, separated by a 1.0 kbp nonhomologous region. This SSV specific (sis) sequence was shown to be uniquely represented within the normal cellular DNA of diverse mammalian species, including human. By Cot and Tm analysis, the sis gene was shown to possess a very close degree of sequence homology with woolly monkey cellular DNA. Thus, our results demonstrate that this primate transforming retrovirus arose in nature by recombination of a type C helper virus and a woolly monkey cellular gene.

Nucleotide sequence analysis is a very powerful tool for analysis of structural genes and their control regions. Methods have been standardized for nucleotide sequence analysis of DNA molecules. The three methods include Sanger's dideoxy method, Maxam and Gilbert's chemical cleavage method and Matt and Smith's nick-translation method. Using a combination of these three techniques, nucleotide sequence analysis of the Moloney murine sarcoma virus (MSV), cloned in E. coli using λ phage Charon 21A vector by scientists within the LCMB, was undertaken. The entire genome of Moloney murine sarcoma virus has been sequenced. Nucleotide sequence analysis demonstrates that the 5817 bp viral genome has two large terminal repeats of 584 bases (LTRs) at both 5' and 3' ends of the proviral genome. The LTRs were found to contain promoter-like sequences, as well as mRNA capping and polyadenylation signals. In addition, they possess a 11-base inverted terminal repeat at each end. Thus, the structure of M-MSV genome with a LTR at each end resembles that of prokaryotic transposable elements. In addition, sequence analysis has revealed that the viral genome has the coding potential for the entire Moloney murine leukemia virus (MuLV) *gag* gene. Both *pol* and *env* genes have suffered large deletions, accounting for the inability of the virus to sythesize either protein product. The transforming region of MSV contains a large open reading frame which encompasses its entire cellular insertion sequence. This open reading frame has initiation and termination points within helper viral sequences. Comparison of the nucleotide sequence of the MSV genome with the known type C viral *gag* gene order and available amino acid sequence data on *gag* gene structural proteins leads to the following conclusions: 1) Both amino-terminal and carboxy-terminal ends of the viral *gag* gene are processed. 2) p15, p12, p30, and p10 polypeptides are coded contiguously. 3) The polymerase is coded within the same reading frame as that of the *gag* gene product.

Finally, comparison of the M-MSV sequence with data obtained so far for A-MuLV shows the presence of certain "hot spots" in the type C RNA viral genome that appear to promote recombinational events leading to the generation of transforming retroviruses.

In vitro translation of virion RNA of Moloney murine sarcoma virus (MSV) *strain 124* yielded major products having molecular weights of 63,000 (63K), 43K, 40K, 31K, and 24K daltons. A molecularly cloned subgenomic fragment of Moloney MSV comprised of the cellular insertion (src) region was utilized in hybridization arrest translation as a means of identifying products of the MSV *src* gene. MSV *src* DNA specifically inhibited synthesis of the 43K, 40K, 31K, and 24K proteins, implying that each of these proteins originated from within the MSV *src* gene. The MSV *src*-specific nature of this family of proteins was further confirmed by partial purification of MSV *src*-containing RNAs from MSV nonproducer cells. *In vitro* translation of enriched cellular RNAs yielded products with molecular weights identical to those of the 43K family of proteins synthesized from virion RNA. Comparison of the molecular weights of the four largest proteins that could be synthesized within the open reading frame of the MSV *src* gene indicated a close correspondence with the molecular weights of the 43K family of proteins. Partial cyanogen bromide cleavage of each of the three largest proteins resulted in an uncleaved fragment having a molecular weight equal to that of the smallest (24K) protein. These findings provide direct biochemical evidence that the 43K, 40K, 31K, and 24K proteins are related in their carboxy-terminal regions, as well as information concerning the MSV *src* gene coding sequences from which each protein originates. Efforts are currently under way to prepare antibodies against peptides synthesized on the basis of nucleotide sequence analysis of the MSV *src* gene open reading frame. It is hoped that antibodies prepared in this manner will aid in the identification of a functional MSV transforming gene product *in vivo*.

LCMB scientists have continued studies aimed at characterization of independent sarcoma virus isolates of the outbred cat. Studies by the Molecular Biology Section have shown that the Snyder-Theilen (ST), Gardner-Arnstein (GA) and McDonough (SM) strains of FeSV, like other mammalian transforming oncoviruses, were replication-defective, requiring a type C RNA virus as a helper.

Studies by LCMB scientists have shown that feline embryo fibroblasts, as well as feline cells of epithelial or lymphoid origin, express low levels of a protein, NCP 92, antigenically related to the sarcoma virus-specific domain of ST-FeSV transforming protein, ST P85. Normal cellular proteins cross-reactive with ST P85 were also detected in cell lines from various other mammalian species. These results suggest that the ST-FeSV sequences encoding for the sarcoma virus-specific domain of ST P85 originated from an evolutionarily conserved cellular gene expressed in cells of independent differentiation lineage.

The transforming protein of ST-FeSV, ST P85, possesses an associated protein kinase activity that specifically phosphorylates tyrosine residues. The physiological significance of this finding is illustrated by the fact that phosphotyrosine is an intrinsic component of ST P85. Furthermore, 5- to 10-fold higher levels of this unusual phosphorylated amino acid were present in ST-FeSV transformants than in uninfected control cells. Thus, phosphorylation of tyrosine residues appears to be associated with cellular transformation caused by Rous sarcoma virus and Abelson murine leukemia virus. These findings suggest that independent transforming virus isolates from birds, mice, and cats may utilize common pathways in exerting their ocogenic potential.

The major gene product of the Gardner-Arnstein (GA) strain of feline sarcoma virus (FeSV) has been characterized. This protein, designated GA P95, is a polyprotein that contains the amino-terminal moiety of the FeLV gag gene-coded precursor protein fused to a sarcoma virus-specific polypeptide. The latter appears to be highly related to the corresponding region of ST P85. Antibodies directed against GA P95 recognized the same feline cellular protein NCP 92 that cross-reacts with ST P85. In addition, GA P95 also has an associated protein kinase activity specific for tyrosine residues whose properties closely resembled those observed in ST P85.

We have studied the humoral immunity against the GA-FeSV gene product in natural FeLV-exposed and experimentally FeSV-inoculated house cats. We found that GA P95 was immunoprecipitated by GA-FeSV immune cat sera, but not by any of the natural FOCMA-immune cat sera tested, after extensive absorption of anti-FeLV antibodies. These results suggest that FOCMA, as identified by immunofluorescence on FL74 lymphoid cells, is not identical to the FeSV transforming protein.

DNA transfection techniques have been utilized to determine the regions of the ST-FeSV gneome involved in malignant transformation. We have found that the 3.7-kbp 5'-end fragment of the ST-FeSV provirus is sufficient to transform NIH/3T3 fibroblasts. Enzymes that cleave the ST-FeSV provirus DNA within the feline leukemia virus gag gene sequences or within the fes gene abolished the transforming activity. Preservation of the proviral large terminal repeats was also required for transformation. Transformed NIH/3T3 cells obtained by transfection of total or subgenomic ST-FeSV DNA expressed normal levels of the ST-FeSV gene product ST P85 and of its associated protein kinase activity. Furthermore, these cells contained high levels of phosphotyrosine residues, a biochemical marker associated with cellular transformation induced by certain retroviruses including ST-FeSV. These results, taken together, strongly support the concept that only those ST-FeSV proviral sequences necessary for ST P85 expression are involved in malignant transformation.

The transformation-specific proteins of ST- and GA-strains of FeSV were shown to be immunologically related to those of the Fujinami and PRC II strains of avian sarcoma viruses. The antigenic determinants shared by these viral proteins have been mapped within their respective sarcoma virus-specific region, suggesting that the cellular insertion sequences present in these avian and feline sarcoma viruses are related. These observations indicate that potentially oncogenic sequences have been conserved during the evolution of feline and avian genomes and have been independently acquired by two sets of sarcoma viruses.

Oncoviruses have been shown to be etiologically involved in naturally occurring tumors of a wide variety of vertebrate species. They appear to exert their oncogenic potential under conditions in which there is poor host cell control of virus replication. This is the case for horizontally transmitted oncoviruses of birds, rodents, arlyodictyles, carnivores and primates. It has become evident that oncoviruses can interact with their hosts in a manner which appears to be unique among viruses of vertebrates. In many species, including primates, oncoviruses are transmitted from one generation to the next, often in an unexpressed form, within the host cell genome. Under such conditions, these endogenous viruses appear to be subject to regulatory processes analogous to those affecting cellular genes. Over the past several years, our laboratory has isolated a number of new endogenous oncoviruses. Moreover, we have investigated the relationships of such viruses to known oncoviruses, as well as the distribution of related viral sequences within vertebrate cellular DNAs by molecular hybridization techniques.

We have applied radioimmunological techniques to demonstrate evolutionary linkage among distantly related oncoviruses by radioimmunological techniques. Antibodies elicited against proteins of a given virus often bind analogous proteins of oncoviruses isolated from species belonging to different Orders or Families of mammals. The designation "interspecies" has been applied to antigenic determinants recognized by an antibody in this kind of reaction. Broadly reactive interspecies antigenic determinants have been demonstrated in the major structural proteins of all known mammalian type C viruses. These findings have been extended to include other translational products representing more than two-thirds of the type C viral coding capacity. All of these studies have led to the conclusion that mammalian type C viruses arose from a common progenitor.

During the past year, collaborative studies involving scientists within the Molecular Biology Section have shown that the major core protein (p28) of MMC-1, an endogenous type C virus of rhesus monkey (*Macaca mulatta*), exhibits extensive homology in its NIH$_2$-terminal amino acids to the sequences of the major structural proteins (p30) of known mammalian type C viruses. Similarly, interspecies antigenic determinants shared by all the above viral proteins were detected in MMC-1 p28. Competition radioimmunoassays, together with the results of statistical analysis of the primary structure data, provided evidence that MMC-1 p28 is not more closely related to primate type C viruses of the Papio genus than to those isolated from rodents, cats or New World monkeys. MMC-1 p28 was found to be closely related, structurally, to the p30 protein of the avian reticuloendotheliosis virus (REV-A), a horizontally transmitted type C virus of putative mammalian origin. In addition, MMC-1 p28 and REV-A p30 shared a specific subset of antigenic determinants not present in any of the other avian or mammalian type C viruses studied. These findings suggest that MMC-1 and REV may have a commmon evolutionary origin.

Research within the Molecular Biology Section is also aimed at elucidating how leukemia viruses cause malignancies. Mouse leukemia viruses have been reported to induce tumors involving cells within the T lymphocyte lineage. We found striking differences in the target cells for *in vivo* transformation by two clonal replication-competent type C viruses, Moloney- and Rauscher-MuLV. Moloney-MuLV-induced tumors and lymphoma cell lines exhibited Thy-1 antigen in the absence of detectable Fc or C3 receptors, indicating their T cell origin. However, Rauscher-MuLV primary tumors and lymphoma cell lines of the same mouse strain invariably exhibited Fc receptors in the absence of Thy-1 antigen, suggesting that these tumors were of the B lymphoid cell lineage. The pattern of immunoglobulin synthesis by individual Rauscher-MuLV tumor cell lines was determined both by biosynthetic and radioimmunologic techniques. Rauscher-MuLV lymphoma lines most frequently expressed immunoglobulin heavy (μ) chain in the absence of detectable light (μ or κ) chains. All of these findings established that the target of neoplastic transformation response to Rauscher-MuLV is an immature cell within the B lymphoid lineage.

To investigate the basis for their cell specificity, we have molecularly cloned these viruses, analyzed their molecular organization and attempted to construct recombinant viruses for biologic testing. The physical map obtained for integrated Moloney MuLV DNA, molecularly cloned in its Hind III permuted form, was consistent with that previously published (Gilboa *et al.*, 1979). We cloned the Rauscher-MuLV genome in its integrated form. Cellular DNA was isolated from a clonal line of NRK cells productively infected with Rauscher-MuLV and digested with Eco RI. This enzyme was previously shown not to cleave the unintegrated linear Rauscher-MuLV genome. Rauscher-MuLV-specific DNA was enriched by RPC-5 column chromatography and sucrose density gradient and used for cloning in lambda phage Charon 4A. The cloned molecule is approximately 14 kbp in length. It was shown to contain an approximate 9 kbp viral DNA, as well as host flanking sequences. The cloned DNA induced virus production in NIH/3T3 cells upon transfection. Restriction enzyme analysis showed a strong correlation between the maps of cloned Rauscher-MuLV DNA and unintegrated linear Rauscher-MuLV DNA. Detailed restriction enzyme mapping of the cloned integrated Rauscher-MuLV DNA was performed. Efforts are now under way to construct recombinant viruses between Rauscher and Moloney-MuLV genomes in order to map the region of the viral genome responsible for target specificity for transformation.

An important genetic approach for analysis of the viral gene(s) involved in leukemogenesis might result from the generation of recombinants between an oncogenic mouse type C virus and an endogenous virus without demonstrable malignant potential. Recombinant viruses were generated in tissue culture between Rauscher-murine leukemia virus (MuLV) temperature-sensitive (ts) mutants restricted at different steps in virus replication and a mouse endogenous xenotropic virus, BALB:virus-2. Mutants utilized included ts 28, a late mutant which releases noninfectious viruses at 39°, and ts 29, a double mutant with a ts lesion in its reverse transcriptase and a late block affecting virus budding. Immunological typing of the translational products of clonal recombinant viruses made it possible to establish their partial genetic maps and localize regions of the viral genome affected by different ts lesions. Recombinants involving Rauscher-MuLV ts 28 invariably contained BALB:virus-2 p15, p12 and p30 proteins, localizing the late defect in replication by this mutant to the 5' moiety of the viral *gag* gene. All ts 29-derived recombinants contained the entire BALB:virus-2 *gag* and *pol* genes. Substitution of the *pol* gene is in agreement with the reported thermolability of Rauscher-MuLV ts 29 reverse transcriptase (Tronick *et. al.*, *J. Virol.* 16: 1476-1482, 1975). Substitution of the *gag* gene suggests that internal structural proteins are actively involved in the virus budding process. Rauscher-MuLV recombinants were utilized to establish the genetic map of the Rauscher-MuLV genome by ologonucleotide T_1 fingerprinting analysis. Detection of Rauscher-MuLV T_1-oligonucleotides in representative recombinant viruses, whose protein phenotypes were established by immunologic techniques, permitted their assignment to specific regions of the viral genome. The genetic map of Rauscher-MuLV generated in these studies should be useful in the identification and characterization of the viral gene(s) involved in leukemogenesis.

Nonadherent tissue culture cell lines were established from normal bone marrow of a variety of mouse strains. The lines possessed morphological and histochemical markers of the basophil/mast cell and contained committed stem cells for metachromatic cells. Their derivation from normal marrow and their lack of tumorigenicity despite long-term culture makes these cell lines potentially important for studies of the mechanisms of allergic reactions and inflammation, as well as the differentiation pathways involving this subset of hematopoietic cells.

As we have developed new biological, biochemical and immunologic techniques for detection of oncovirus expression, these techniques have been applied to the search for oncovirus expression in man. For example, we have developed very broadly reactive competition radioimmunoassays that detect most, if not all, known oncoviruses. These assays might be expected to be useful in the detection of antigenic determinants of new oncoviruses. Similarly, the analysis of new virus isolates has been aided by the development of highly type-specific radioimmunoassays that can discriminate between very closely related virus strains. Immunoassays have also been applied to epidemiologic studies, in which we have searched for antibodies to oncoviruses in man. The recent development of molecular clones of the cell-derived transforming regions of a number of transforming retroviruses has made it possible to utilize these defined genetic segments as molecular probes to search for related transcripts in human malignancies. Preliminary evidence already indicates that transcripts related to the transforming regions of SSV and MC-29 viruses are expressed specifically and at high levels in certain human tumors. The possibility that these transcripts are etiologically related to human neoplasia is being actively explored.

The Experimental Oncology Section has been involved in both immunologic and molecular studies of carcinomas, with emphasis on mammary neoplasia. The molecular studies of the murine mammary tumor model have elucidated the broad spectrum of both genetic elements and mechanisms that may be involved in the etiology of a carcinoma of a given species. Molecular studies by the Experimental Oncology Section and others have shown that several different MMTV variants may be involved in this process. The large number of copies of MMTV present in normal mouse cells have made it difficult to precisely define the mechanism by which MMTV transforms mammary epithelial cells. Attempts have thus been made to correlate the presence of specific MMTV proviral variants with the incidence of mammary tumors in mouse strains with a low, moderate or high incidence of mammary cancer, as well as in feral, outbred populations of the genus *Mus*. C3H/StWi mice have a low incidence of spontaneous mammary tumors. In spite of the fact that four distinct endogenous MMTV proviruses have been identified in this strain, only 7 out of 441 breeding females developed mammary tumors. To determine which, if any, of the endogenous proviruses was involved in inducing the mammary tumors, high molecular weight DNA from normal C3H/StWi tissue was digested with specific restriction endonucleases and the MMTV-specific DNA fragments identified by hybiridization to MMTV-cDNA. These studies have shown that if MMTV is involved in the induction of spontaneous or chemically induced C3H/StWi mammary tumors it does so by mechanism other than the simple amplification of pre-existing endogenous MMTV genes. This is the first system in which spontaneous murine mammary tumors have been shown to arise without the presence of amplified MMTV proviral information.

Although the mammary tumor incidence in most strains of BALB/c mice is low, over 50% of the breeding females of the BALB/cV strain develop mammary tumors by the age of 10 months. To determine if any alteration in the MMTV proviral information of these mice was involved in this dramatic increase in tumor incidence, we examined the MMTV proviral DNA content of both normal and tumor tissue of BALB/cV mice. In addition to endogenous MMTV-specific bands found in all organs of BALB/cV mice, two additional MMTV-specific restriction fragments in DNA from BALB/cV mammary tumors were identified. These fragments appeared to contain the genetic information for an infectious MMTV provirus that could be distinguished from any of the endogenous BALB/cV proviruses. To date, the additional MMTV proviral information found in mammary tumors of BALB/cV mice can be distinguished from any endogenous or exogenous MMTV variant reported thus far. In fact, we have been able to isolate an infectious virus from the BALB/cV strain of mice. This new MMTV variant has been shown to be oncogenic for BALB/c mice and also immunologically distinguishable (using monoclonal antibodies) from other infectious variants of MMTV.

The GR strain of mice is interesting in that over 95% of breeding females develop mammary cancer before 1 year of age. Traditionally, the MMTV has been thought to be involved in the induction of mammary tumors in GR mice. However, the mechanism by which MMTV might induce mammary cancer in the strain is unclear since both liquid hybridization studies and restriction endonuclease analysis has identified five endogenous MMTV proviruses in normal GR tissues. To identify which endogenous GR provirus(es) is involved in the induction of mammary cancer, crosses were set up between GR mice and C57BL mice in which the incidence of mammary cancer is 0%. In BC1 females [C57Blx(C57BlxGR)] mammary tumor incidence segregated as a single Mendelian unit in that 50% of the animals developed mammary cancer. We have developed three molecular markers for a single endogenous GR provirus which segregate as a single Mendelian unit with the occurrence of mammary cancer in these mice. The markers are (1) MMTV-specific Sac 1 restriction fragments of 6.2, 2.2, and 0.9 kbp, (2) MMTV-specific Bgl II retriction fragment of 4.3 kbp and (3) a subset of nucleic acid sequences of the RNA genome of the highly oncogenic MMTV(C3H) called tumor-associated sequences. Thus we have identified a single endogenous GR provirus which appears to be involved in the induction of mammary cancer in GR mice.

Studies were also undertaken to elucidate the genetic relationship between the genome of a novel retrovirus (designated M432) isolated from M. cervicolor and the intracisternal type A particle (IAP) genome. The M432 retroviral genome was molecularly cloned in bacteriophage lambda and the nucleotide sequence of the long terinal repeat (LTR) of the M432 genome was determined. Comparative analysis of the M. musculus IAP and M432 retroviral genomes revealed two regions of sequence homology. The sequences which are unique to the M432 viral genome are not well conserved in species other than M. cervicolor, whereas the IAP-related sequences were shown to be highly conserved in all murine species.

In complementary studies by the Experimental Oncology Section aimed at identifying MMTV and MMTV tumor-associated antigens, mice and rats were immunized with disrupted MTVs from M. musculus (MMTV[C3H]), M. cervicolor (MC-MTV) and M. cookii (MCo-MTV), One monoclonal antibody bound to the highly oncogenic MMTVs from C3H and GR mice, but not to the highly oncogenic MMTVs from RIII and A mice. This monoclonal was also able to distinguish between two MMTVs with moderate oncogenicity, i.e., it bound to the MMTV purified from BALB/c mice, but not MMTV from C3HfC57BL mice. In fact, MMTVs from six different strains of M. musculus could clearly be distinguished from each other by this method. By immunoperoxidase technique, the monoclonal antibodies generated were used to detect MTV antigenic determinants on tissue sections of primary and transplanted murine mammary tumors. Using these antibodies to monitor expression of a distinct antigenic determinant in tumor cells, it became apparent that a given antigenic determinant can be expressed in a different manner in two different mammary tumors. The second phenomenon was the quantitative heterogeneity in staining within a given mammary tumor, i.e., most mammary tumors tested from a variety of Mus strains and species presented some areas that were positive, and some that were negative, for expression of a given determinant.

Another major area of investigation within the Experimental Oncology Section relates to a generation of monoclonal antibodies directed against human mammary cancer. Monoclonal immunoglobulins from eleven hybridoma cell lines prepared following immunization of mice with membrane enriched fractions of human metastatic mammary carcinoma cells have been analyzed. Ten were IgG of various subclasses and one was an IgM. The primary screen for monoclonal antibodies reactive with human mammary carcinoma cells was a solid phase RIA employing cell extracts of two breast tumor metastases and apparently normal human liver as test antigens. All eleven antibodies were negative when tested against similar extracts from: normal human liver, rhabdomyosarcoma cells, a cell line derived from cultures of human milk, a mouse mammary tumor or fibroblasts, and MMTV or MuLV.

Each antibody was also tested for binding to live cells in culture. Test cells included three estalished cell lines of human mammary carcinoma, several cell lines of other human tumors, and eleven cell lines established from apparently normal human tissues. The nine monoclonals grouped together on the basis of their binding to both metastatic cell extracts could be separated into three different groups on the basis of their differential binding to the surface of live mammary cells in culture. None of the antibodies bound to several sarcoma and melanoma cell lines tested. Some of the antibodies appeared to possess a "pancarcinoma" pattern of binding activity. Two of the monoclonals, on the other hand, did not react with the surface of any of the mammary tumor cell lines tested, but could be distinguished from the other nine monoclonals by their differential binding to cell extracts. None of the eleven monoclonal antibodies bound to cell lines derived from apparently normal human tissues. To further define the range of reactivity of each of the eleven monoclonal antibodies, the immunoperoxidase technique on tissue sections was employed. All the monoclonals reacted with mammary carcinoma cells of primary and metastatic mammary carcinomas. A high degree of selective reactivity was observed with mammary tumor cells, and not with apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes of the breast.

In other approaches, lymphocytes from lymph nodes obtained at mastectomy in breast cancer patients have been fused with murine non-Ig-producer myeloma cells to obtain human-mouse hybridoma cultures that synthesize human monoclonal antibodies. To date, over 1,400 microtiter wells have been seeded with fusion products of murine NS-1 myeloma cells (non-Ig secreting) and lymphocytes from 16 patients. Hybridoma cultures were first tested for the synthesis of human IgG or IgM 1,428 days after fusion. Of the 301 replicating hybridoma cultures, 52 (17%) synthesized either human IgG or human IgM. The duration of human Ig synthesis ranged from 14 to at least 300 days. Twenty-three of the 52 cultures (44%) continued to synthesize human Ig through the 61 to 300 days of observation. The titers observed were comparable to those detected in our laboratory, and by others, with both mouse-mouse and mouse-rat hybridomas.

Human monoclonal antibody MBE6 was first tested by immunoperoxidase for reactivity with tissue sections of the primary breast tumor mass (infiltrating duct carcinoma) of patient MB and demonstrated marked cytoplasmic staining differentiation or malignancy of a given mammary tumor cell population. These monoclonals may also serve a useful purpose in providing a marker for the transformed state in *in vitro* carcinogenesis experimentation. If monoclonal antibodies are ever used clinically, human monoclonal antibodies may have the advantage over murine monoclonal antibodies in that one would anticipate a reduced immune response to human immunoglobulin. It is hoped that the murine and human monoclonal antibodies generated and characterized by the Experimental Oncology Section to date may eventually have application in the diagnosis, prognosis, and treatment of human neoplasms.

LCMB efforts to elucidate mechanisms of carcinogenesis are complemented by the In Vitro Carcinogenesis Section. Research of this Section is aimed at determining mechanims of spontaneous and carcinogen-induced malignant transformation of cultured cells of rodent and human origin. Special emphasis is being directed at the development of culture systems utilizing human epithelial cells to study the interactions of chemical carcinogens and viruses with cellular DNA. Further efforts are aimed at defining the fundamental cytologic, biologic, and biochemical characteristics of carcinogenic change.

One achievement of the past year is the development of quantitative procedures for more accurate measurement of growth responses in human keratinocytes in culture. With these procedures for preparation of replicate cultures and measurement of cell growth by enumeration of isolated cell nuclei, we have determined the calcium level (1 mM) that yields maximal cell proliferation in mass culture under our conditions, a level higher than previously reported by other investigators examining requirements for colony growth. Calcium effects on morphology of human keratinocytes have also been documented, i.e., an increasing degree of compactness of colony and cell density with increasing Ca^{++} concentration. With our larger yields of cells, we are currently applying various protocols for inducing neoplastic transformation.

Visible light and oxygen are ubiquitous environmental agents which interact with cells in culture and their surrounding milieu. Solar energy peaks in the visible range and radiant energy in the 400-500 nm band are greater than in any subsequent 100 nm band. Although skin filters ultraviolet light, significant amounts of visible light penetrate superficial tissue layers. Visible light has been shown to be mutagenic, to promote neoplastic transformation in vitro and to produce chromatid breaks and exchanges and DNA-protein cross-links in both mouse and human cells in culture. Skin epithelial cells may have more protective defense mechanisms against visible light than fibroblasts. Normal human fibroblasts illuminated in medium NCTC 168 are rapidly killed, whereas primary foreskin epithelial cells appear unaffected. The effective wavelength for this response is in the near visible range (365-405 nm). Illumination of cell-free NCTC 168 by fluorescent light generates stable cytocidal photoproducts, including hydrogen peroxide (H_2O_2), and 60% of the cell killing is prevented by catalase. By using exogenous H_2O_2 and DNA damaging drugs, DNA strand breakage, DNA repair and cell survival are being monitored in these cell systems.

Another aspect of the Section program concerns the repair of DNA damage inflicted by low-level (non-toxic) insults to DNA. One lesion of current interest is the DNA-protein cross-link which is induced by a wide variety of carcinogenic agents including x-rays, light, and many chemicals. Although thought to be non-mutagenic, DNA-protein cross-links are reported to increase sister-chromatid exchanges and to transform 10T-1/2 mouse cells. A major finding this year is that the repair of DNA-protein cross-links induced in mouse cells by the reagent trans-Pt (trans-diaminodichloroplatinum) is cell-cycle dependent and not dependent on the cycle-independent excision repair systems nor on the caffeine-sensitive postreplication repair mechanism. Thus, in noncycling tissues this type of damage could accumulate with time and have marked epigenetic effects at the level of induction of protein and production of messenger RNA. Another finding of importance is that trans-Pt, as compared with cis-Pt, reacts much faster to form DNA-protein cross-links and in the absence of protein may form DNA-DNA cross-links faster than cic-Pt. The toxicity and antitumorigenic activity of cic-Pt, widely used today in chemotherapy, is thought to be due to its ability to form DNA-DNA cross-links. This property is attributed to a steric configuration which inhibits DNA-DNA interstrand cross-link formation by trans-Pt. The discovery of the greater reactivity of trans-Pt suggests an alternative explanation. The first reactive chloride of trans-Pt would be largely displaced before reaching the DNA of a cell. Since in cis-Pt both chlorides react slowly, there is a much higher probability that unreacted cis-Pt will reach DNA in the nucleus before any reaction, and thus allow both groups on one Pt to react with DNA to form interstrand cross-links. This concept may be useful in optimizing the design of Pt compounds for chemotherapy.

Other studies in this Section are concerned with the role of DNA damage and deficient repair in the process of malignant transformation. We reported previously that mouse cells after spontaneous malignant transformation in culture show a significant increase in susceptibility to chromatid damage produced by a single exposure to low-intensity fluorescent light. The effective wavelength (405 nm) is in the visible range. Furthermore, the chromatid damage, including breaks and exchanges, results directly or indirectly from the intracellular generation of clastogenic amounts of H_2O_2 and the derivative free hydroxyl radical (·OH) since damage can be almost completely prevented by adding catalase and/or mannitol, a scavenger of ·OH, to the culture medium during light exposure. We obtained cytogenetic evidence for two defects in DNA repair capacity in the malignant mouse cells as compared with their normal counterparts, i.e., a deficiency in a caffeine-sensitive mechanism, presumably post-replication repair, during S-phase and deficiency in a caffeine-sensitive mechanism operative during late S-G_2. Like mouse cells, skin fibroblasts from individuals with xeroderma pigmentosum and ataxia telangiectasia, genetically predisposed to a high risk of cancer, showed an increase of susceptibility to light-induced chromatid breaks following culture. The level of susceptibility was significantly higher than attained in 13 lines of fibroblasts from normal skin of donors ranging in age from fetal to 92 years and tested after various periods of culture. Also, normal human skin fibroblasts transformed by chemical carcinogens to tumorigenic cells showed a significant increase in susceptibility as compared with their normal controls. These data indicated for human cells, as for mouse cells, an association between enhanced susceptibility to light-induced chromatid breaks and neoplastic potential, an association further supported by the high susceptibility of cells derived from a human adenocarcinoma and recently of cells from other human tumors.

Increased susceptibility to chromatid breaks could result from greater initial chromosomal DNA damage, or from impaired capacity to repair the damage. Greater initial damage could result from loss or inactivation of defense enzymes such as glutathione peroxidase (GSH:peroxidase) which scavanges H_2O_2. Activities of GSH:peroxidase were similar in the paired normal and tumorigenetic human cell populations. Also, cells of the paired lines were equally sensitive to DNA breakage by exogenous H_2O_2. These observations suggested that the enhanced susceptibility associated with neoplastic potential results from an impaired capacity to repair DNA rather than a greater initial sensitivity of the neoplastic cells to the damaging agent.

Investigations by the LCMB over the past several years have suggested that prevention of spontaneously and chemically induced cancers in animal model systems might be accomplished by active or passive immunization with appropriate preparations specific for tumor cell antigens held in common by various neoplasms of a given species. Efforts have focused on the determination of which endogenous viral gene products might be the most effective immunogens, utilizing tumor prevention as the end point. Achievement of this goal not only fulfills the pragmatic objectives of cancer prevention but at the same time may provide important insights into mechanisms involved in the causation of cancer. These efforts have broadened in scope to include the search for shared tumor antigens that may be coded by endogenous transforming (src) genes to development of simple and reproducible skin transformation assays for detection of individuals at high risk for cancer, and efforts aimed at immunotherapy of primary and metatastic cancer in naturally occurring animal tumor systems.

During the past year, studies demonstrating immuoprevention of 3-MC-induced cancers in mice (C57BL/6 and NIH Swiss) and rats (Fischer 344) were extended and confirmed, with significant protection demonstrated by the use of retroviruses bearing appropriate antigenic determinants. The retroviral and cell vaccines which were successful included the following: In the C57BL/6 mouse, RadLV, the endogenous retrovirus of the C57BL/6; in the NIH Swiss mouse, AKT-8, a mink cell line transformed by the AKR virus, another endogenous mouse virus. In the rat system, only RaLV, the endogenous rat virus, and the AT-124, an endogenous mouse virus with shared determinants with the rat virus, were protective. These studies support the role of viral oncogenes in both cancer cause and prevention.

Studies were also performed to treat spontaneous and induced animal cancers with retroviruses derived from a variety of mammalian systems, including the baboon, cat, squirrel monkey, laboratory and wild mice. Retroviruses chosen had been demonstrated to replicate well in the respective host cells, but lacked oncogenicity in test animals. The rationale behind this approach was the knowledge that the retroviruses grow preferentially in tumor tissue; the infected tumor cells were thus rendered highly antigenic, stimulating a powerful cell-mediated immune rejection.

The host chosen as the nearest freely available surrogate to the human was the dog, which has no known retrovirus of its own, develops spontaneous cancer at approximately the same incidence levels and of the same cell types as the human, and can be evaluated and treated in veterinary hospitals. Treatment regimens included inoculation of appropriate retroviruses intravenously, intraperitoneally, directly into the tumor site(s), and/or combined routes. The viruses were of high titer (10^4 - 10^6) and were used either unconcentrated or concentrated at least 1000X. Dogs on test had sometimes undergone prior chemotherapy and/or surgery. In every case, the cancers were confirmed clinically and pathologically, sometimes several times. There were some successes. Eight dogs are in long-term remission. In addition to the eight, one which had appeared "cured," died 11 months after its last treatment (at 13 years of age) with residual traces of its original hemangiosarcoma, suggesting the need for precautionary follow-up "booster" treatments.

Human skin fibroblasts derived from cancer family syndromes (Gardner's syndrome, FAMMM and ACR), passaged 3-7 times, were tested for focus formation using the KiMSV or KiMSV (baboon) viruses in three laboratories (Drs. M. Gardner and S. Rasheed, USC; Dr. L. Kopelovich, Sloan-Kettering, and Drs. R. Huebner and J. Rhim, LCMB). In each of the laboratories, the individuals of such cancer families bearing autosomal dominant genes specifying early cancer were clearly identified. Biopsies derived from individuals at risk required 10^4 - 10^5 virus titers. Confirmatory efforts are underway in the same laboratories with 40 additional specimens provided blindly by Dr. Eldon Gardner from his large repository at the University of Utah. When tests have been completed sometime this year, the code will be broken. In other related collaborative studies with Dr. J. Frankel (State of Florida, Dept. of Health, Tampa), biopsies derived from heavy smokers and others known to be heavily exposed to environmental carcinogens are being tested to determine if these assays can identify individuals likely to develop cancer because of certain long-term environmental exposures. Tests are now being designed for use in smokers with and without lung cancer. When predictable test parameters have been established and confirmed, these assays promise to have diagnostic and prognostic importance.

The objectives of the Experimental Ontogeny Section center on understanding the significance of interferons (IFN) in placental tissue extracts and their relationship to retrovirus expression during prenatal ontogeny. The IFN's are a class of low molecular weight glycoproteins that potentiate many diverse types of biological actions. In addition to their antiviral activity, IFN's influence a variety of other physiological processes ranging from cell surface and growth control to immunoregulation. Indeed, interferons are regarded as one of nature's most potent and best characterized negative growth regulators, and presently, a major effort is in progress to evaluate their therapeutic value in cancer treatment. The demonstration of interferons, as well as other growth regulating components, in placentas--an organ immunologically foreign to and protected from rejection by its host, as well as possessing numerous morphological, immunological, endocrine and biochemical similarities to a variety of cancers--provides a unique model system to investigate growth regulation and the role of interferon in cellular recognition and differentiation.

During the past year, the appearance of a pregnancy-dependent antiviral activity, exhibiting biological, immunological and biophysical characteristics of IFN's, in placental extracts of mice was demonstrated. The antiviral activity was shown to be protein associated (trypsin sensitive) and to require a cellular transcriptional event (actinomycin D sensitive). Additionally, the activity was neutralized by antibody to L-cell IFN, was partially resistant (≈20%) to acid treatment (pH 2.0/4°C for 72 hrs) and exhibited species specificity. Preliminary characterization of the antiviral activity by gel filtration indicates the presence of at least two components, one of which migrates in the molecular weight region of virus-induced (Type 1) IFN. The level of placental IFN (IFN-P1) is dependent ($P < 0.5$) on the stage of gestation and genetic background. Strains exhibiting the highest mean IFN-P1 levels at term included C_{57}Bl/6N, C_3H/HeN and N.NIH(S). By comparison, IFN-P1 titers of NZB/N, BALB/cAnN, AKR/N and DBA/2N were lower ($P < 0.05$) and clustered around a mean of 1000 units/ml. In all mouse placentas examined, IFN-P1 was detected as early as day 13 of gestation. Although at this stage of pregnancy IFN-P1 levels were 2- to 3-fold lower than at term ($P < 0.05$), differences among strains were already evident ($P < 0.05$). These differences parallel the reported ability of these strains to produce IFN in response to viral and immunological induction. Although the inductive event(s) of IFN-P1 has not been established, its level is dependent on the genotype of the maternal parent and appears to involve an adherent cell population.

The presence of mitogenic (MSF) and colony (CSF) stimulating factors in term placental extracts was also established. In contrast to the properties of IFN-P1, however, MSF activity does not manifest species specificity and is highly stable to low pH treatment. The mitogenic activity is associated with components exhibiting molecular weights of approximately 15,000 and 70,000 with the former showing the greater activity. The relationship of CSF to MSF is currently under investigation.

During the past year, the LCMB initiated a program within the Office of the Chief aimed at the isolation and characterization of the human gene for cytochrome P-450, a group of NADPH- and/or NADH-dependent membrane bound multicomponent enzyme systems, involved in metabolism of steroids and biogenic amines. Messenger RNA isolated from 3-methylcholanthrene-treated C57BL/6N mice was purified by oligo dT-cellulose chromatography and sucrose gradient centrifugation. A double-stranded, complementary DNA copy was synthesized using avian myeloblastosis virus reverse transcriptase and DNA polymerase I. Single stranded ends were removed by S1 nuclease and oligo dC tails added by terminal deoxynucleotidyl transferase. These dC-tailed fragments were then inserted into pBR322 plasmid DNA which had been cut with Pst I restriction endonuclease and tailed with oligo dG. Seventy-two ampicillin-sensitive, tetracycline-resistant clones were analyzed by hybridization to [^{32}P] cDNA synthesized using mRNA from methylcholanthrene-treated C57BL/6N responder mice and DBA/2N nonresponder mice. One clone (clone 47) was found which hybridized to responder but not to non-responder cDNA. The insert in this clone was shown to be 1100 bp in length. In translation arrest experiments, the cloned DNA was bound to diazobenzyloxymethyl (DBM) paper and used to bind mRNA from methylcholanthrene-induced C57BL/6N mice. The bound mRNA was shown immunologically to code for P_1-450 when DNA from clone 47 was bound to the DBM paper but not when DNA from any of the other ampicillin-resistant clones was used.

The human P_1-450 gene has been identified by hybridization of nick-translated clone 47 DNA to Eco RI digested human placenta DNA. Human placenta DNA was first restricted by Eco RI endonuclease and separated on the basis of base composition by reverse phase chromatography on RPC-5. Each of twenty fractions from the RPC-5 column was then electrophoresed in a 1% agarose gel and transferred to DBM paper. Hybridization of this filter to nick-translated clone 47 DNA under relaxed hybridization conditions showed the presence of a 4 kbp sequence which has now been isolated and cloned in Charon 16A. Identification and characterization of phage carrying human P_1-450 genes is currently under way.

In addition to their intramural research efforts, investigators within the LCMB serve on the editorial boards of major journals in their field, serve as members of various grant review bodies, and participate in a large number of collaborative efforts with scientists in laboratories throughout the country. The major goal of studies within the Laboratory is to apply, wherever possible, basic information derived from a multi-disciplinary approach to the study of virus-induced and spontaneously occurring cancers to its most important application, the prevention of cancer in man.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04805-11 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cocarcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Hellman	Assistant Chief	LCMB	NCI
OTHERS:	A.K. Fowler	Head, Experimental Ontogeny Section	LCMB	NCI

COOPERATING UNITS (if any)

Food and Drug Aministration Bureau of Radiological Health Rockville, MD 20857	Litton Bionetics, Inc. Frederick Cancer Research Center Frederick, MD 21701

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is designed to gain further insight into the control of genomic expression, particularly as it may lead to deregulatory processes. Systems consisting primarily of primary rodent and human cultures are being used to evaluate modification of genetic expression based on exposure to tumor promoters (such as chemicals), x-ray and ultraviolet light.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

Tumor promoters are a group of compounds that by themselves do not induce cancers, but markedly enhance the production of malignancies if administered at times after exposure to low doses of carcinogen. The mechanism of initiation and promotion is not well understood. We are attempting to elucidate mechanisms of tumor promotion utilizing immunological and enzymatic techniques.

Methods Employed:

Cell exposure to tumor promoters, U.V. and x-irradiation and evaluation of the subsequent response is being studied *in vitro*.

Major Findings:

The tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induces endogenous murine xenotropic type C retroviruses from A 1-2 cells, derived from the BALB/c mouse, as determined by infectious center focus forming assay on permissive normal rat kidney cells (NRK). Kinetic dose response studies showed that the number of cells induced to release virus was dependent on TPA concentration and time of assay following TPA exposure. Maximal induction occurred when cells were treated with 80 μg/ml of TPA for 24 hr and assayed at 24 and 48 hr. A 30 min pulsed TPA exposure of 200 μg/ml induced virus levels approximating those observed after continuous 24 hr exposure to 80 μg/ml. The combination of TPA concentration of 10 and 20 μg/ml and suboptimal levels of 5-iodo-deoxyuridine (IuDR) enhanced retrovirus induction three-fold above that seen by the optimum IdUrd concentration alone. The protease inhibitors antipain and leupeptin decreased virus induction by TPA, a protease inducer. The capacity of TPA to induce type C retrovirus complements results which demonstrate the enhancement of Epstein-Barr virus and murine mammary tumor virus synthesis by TPA.

Significance to Biomedical Research and the Program of the Institute:

The protease inhibitor data suggests that protein suppressors of virus expression similar to those described for the bacteriophage system may be involved in this mammalian system. A means may therefore be available to study and begin to understand the regulation of endogenous viral expression in a mammalian system.

Proposed Course:

We propose to further illucidate the mechanisms associated with protein repression and derepression. We will also attempt to define certain parameters by which TPA and other tumor promoters bring about their multitude of biological and biochemical consequences.

Publications:

Saviolakis, G.A., Strickland, J.E., Hellman, A., and Fowler, A.K.: Estradiol effect on type C viral gene expression in the uterus of the ovariectomized mouse. Proc. Soc. Exp. Biol. Med. 164: 184-191, 1980.

Strickland, J.E., Saviolakis, G.A., Weislow, O.S., Allen, P.T., Hellman, A., and Fowler, A.K.: Spontaneous adrenal tumors in the aged, ovariectomized NIH Swiss mouse without enhanced retrovirus expression. Cancer Res. 40: 3570-3575, 1980.

Giron, D.J., Patterson, R.R., Hellman, A., and Fowler, A.K.: Enhancement of encephalomyocarditis virus replication in L cells treated with insulin. Proc. Soc. Exp. Biol. Med. 166: 305-309, 1981.

Hellman, K.B., Brewer, P.P., Twardzik, D.R., and Hellman, A.: Protease inhibitors modify tumor virus induction. Proc. Soc. Exp. Biol. Med. 166: 28-34, 1981.

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Weislow, O.S., Fisher, O.U., Jr., Fowler, A.K., Twardzik, D.R., and Hellman, A.: Depression of mitogen-induced lymphocyte blastogenesis by baboon endogenous retrovirus associated components. Proc. Soc. Exp. Biol. Med. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04807-11 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Comparative Analysis of Fetal and Neoplastic Growth Regulating Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. K. Fowler	Head, Experimental Ontogeny Section	LCMB	NCI
Other:	P. T. Allen	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

Wright State University
School of Medicine
Microbiology & Immunology Program
Dayton, OH 45435

Litton Bionetics, Inc
Fred. Cancer Res. Ctr
Frederick, Maryland

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Experimental Ontogeny Section

INSTITUTE AND LOCATION
NCI, NIH Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The induction and regulation of retroviruses in reproductive tissues during pregnancy and their relationship with growth regulating factors during prenatal ontogeny are being examined. Interferons (IFN), mitogenic stimulating factors (MSF) and colony stimulating factors (CSF) have been identified in murine gestational tissues and in vitro model systems developed to characterize the bioregulatory activities of these factors, singularly and in combination. IFN are detected in placentas of fetuses as early as the 13th day of pregnancy, then their levels progressively increase to term. Biophysical characterization of antiviral activity by gel filtration indicates the presence of at least two components, one in the molecular weight region of virus induced (Type 1) IFN. The relative level of placental interferon is under maternal genetic control and its production appears to involve an adherent cell population.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The objectives of this project are to develop and examine model systems for: (1) identifying and characterizing fetal and tumor-associated bioregulatory macromolecules and to assess and compare their interactions in cellular recognition and growth regulation processes; and (2) studying the physiological induction and regulation of endogenous retrovirus expression *in vivo* and their potential influence, directly or indirectly, on normal and pathological growth control mechanisms of the host.

Methods Employed:

Interferons (IFN) in extracts prepared from mouse tissues are assayed by their antiviral [vesicular stomatitis virus (VSV), or encephalomyocarditis virus, strain MM] activity on L_{929} murine cells using plaque reduction techniques (PR_{50}), inhibition of cytopathogenic effect (CPE), virus yield reduction and inhibition of viral specific RNA synthesis. Stimulation of cellular mitogenesis (MSF) by tissue extracts is quantitated by the incorporation of [^{3}H]-thymidine into the DNA of confluent mouse, rat or human tissue culture lines. Colony stimulating factors (CSF) are identified by their growth induction of BALB/3T3 and NRK cells in soft agar. Oncornavirus expression is determined by radioimmunoassay, molecular hybridization, RNA-directed DNA polymerase activity and tissue culture techniques. Cellular receptor x ligand interactions are examined by radiolabeled ligand binding reactions. These assays are used in concert to study the influence of oncornavirus expression and/or the interaction of growth regulating factors on cellular recognition and differentiation processes associated by prenatal ontogeny and tumorigenesis.

Major Findings:

The appearance of a pregnancy-dependent antiviral activity, exhibiting biological and immunological characteristics of interferon, in the gravid uterus and placenta of random-bred N.NIH(S) mice has been described. Interferon was detected in most uterine specimens between 10-15 days of gestation (300 units/ml); however, samples taken before or after this period of pregnancy, as well as from virgin mice, had no detectable antiviral activity (<10 units/ml). In comparison, interferon was detected in all placental samples and its titer increased significantly during gestation (>2500 units/ml). The antiviral activity has been shown to be protein associated (trypsin sensitive) and to require a cellular transcriptional event (actinomycin D sensitive). Additionally, the activity is neutralized by antibody to L-cell interferon, is partially resistant (≈20%) to acid treatment (pH 2.0/4°C for 72 hrs.) and exhibits species specificity; i.e., 24 PR_{50} units placental interferon/ml reduced MM and VSV yields in murine cells (>85%), but not in hamster, mink, bat, or human cells (0%). Preliminary biophysical characterization of the antiviral activity by gel filtration has revealed the presence of at least two components one migrating in the 65-80 kd region and the other in the 15-25 kd region.

More recently, these observations have been extended by examining placental tissue extracts of mice from several different strains for a similar pattern of interferon accumulation, as well as testing these tissues for other growth regulatory factors (MSF and CSF). Similar to that of N.NIH(S) mice, interferon activity was detected in placental extracts of mice of all other strains examined, but its level was dependent ($P < 0.05$) on genetic background. Strains exhibiting the highest mean interferon level (>2000 units/ml) in term placentas (>18 days of gestation) included C_{57}Bl/6N, C_3H/HeN and N.NIH(S). By comparison, placental interferon titers of NZB/N, BALB/cAnN, DBA/2N and AKR/N were substantially lower ($P < 0.05$) and clustered around a mean level of 1000 units/ml. Significantly, the relative ability of strains to produce placental interferon paralleled the reported ability of these strains to produce interferon following either viral or immunological induction. Although the inductive event(s) of gestational interferon has not been established, recent data obtained from reciprocal cross matings of low and high producer strains (BALB/cAnN and C_{57}Bl/6N) suggest its level is under maternal regulation, and furthermore, appears to involve an adherent cell population.

The presence of mitogenic stimulating factors in term N.NIH(S) placental extracts has been established. In contrast to the properties of placental interferon, however, the mitogenic activity does not exhibit species specificity and is stable at low pH treatment. The mitogenic activity is associated with components that migrate in the 70 kd and 15 kd regions during gel filtration and is distinguishable from epidermal growth factor, a potential tissue contaminant, by molecular weight, cell type specificity and cell receptor competition analysis. Of the two components, the major activity appears to be associated with the smaller molecule. Additionally, these placental extracts also exhibit colony stimulating activity and the relationship of this activity to MSF is presently under investigation.

In continuing studies, a pregnancy-associated variation in retrovirus-specific RNA sequences, similar in pattern to that seen for viral-p30 and RNA-directed DNA polymerase, has been demonstrated in uterine tissue homogenates of N.NIH(S) mice. Using molecular hybridization with complementary DNAs synthesized from high molecular weight type C viral RNA (R-MuLV), a rapid decline (≃10-fold) in viral-specific RNA was evident during the first trimester (<8 da), presumably due, in part, to a dilutional effect resulting from rapid decidua formation, a tissue exhibiting little, if any, virus expression. Subsequently, the relative level of uterine viral-specific RNA increased progressively during gestation, attaining concentrations equivalent to non-gravid uteri (0.004% of cellular RNA) near term. In view of reports that retroviruses induce interferon, the role of these viruses in placental interferon induction is being considered.

Significance to Biomedical Research and the Program of the Institute:

The interferons are a class of low molecular weight glycoproteins that potentiate many diverse types of biological actions. In addition to their viral prophylactic activity, interferons influence a variety of other physiological processes ranging from cell surface and growth control to immunoregulation. Indeed, interferons are regarded as one of nature's most potent and best characterized negative growth regulators, and presently, a major effort is in progress to evaluate their therapeutic value in cancer treatment. The demonstration of interferons, as well as other growth regulating components, in placentas--an organ immunologically foreign to and protected from rejection by its host, as well as possessing numerous morphological, immunological, endocrine and biochemical similarities to a variety of cancers--provides a unique model system to investigate growth regulation and the role of interferon in cellular recognition and differentiation.

A comprehensive study of the induction and regulation of growth regulating molecules, and their interactions, in this temporary organ should contribute valuable insight into the understanding of normal and neoplastic development, particularly as it relates to transplacental and prenatal carcinogenesis.

Proposed Course:

The interferons in placental tissues will be further purified by biochemical, biophysical and immunological techniques for a more detailed characterization of their biological activities, singularly and in combination with other fetally derived growth regulating factors. Model systems will be developed to examine these interactions as they relate to prenatal ontogeny and as they are modified by chemical transplacental and prenatal carcinogens. Progress on these objectives has been hampered by the relatively low titer of placental interferon and its lability during processing and storage. Therefore, emphasis initially will be placed on developing buffer systems to optimize extraction efficiency and stabilize activity during purification procedures. Attempts to identify the mechanism of placental interferon induction, the producer cell(s) and site(s) of synthesis will be continued.

Publications:

Allen, P. T., Strickland, J. E., Fowler, A. K., and Waite, M. R. F.: Antigenic determinants shared by the DNA polymerases of reticuloendotheliosis virus and mammalian type C retroviruses. Virology 105: 273-277, 1980.

Fowler, A. K., Reed, C. D., and Giron, D. J.: Identification of an interferon in murine placentas. Nature 286: 266-267, 1980.

Saviolakis, G. A., Strickland, J. E., Hellman, A., and Fowler, A. K.: Estradiol effect of type C viral gene expression in the uterus of the ovariectomized mouse. Proc. Soc. Exp. Biol. Med. 164: 184-191, 1980.

Strickland, J. E., Saviolakis, G. A., Weislow, O. S., Allen, P. T., Hellman, A., and Fowler, A. K.: Spontaneous adrenal tumors in the aged, ovariectomized NIH Swiss mouse without enhanced retrovirus expression. Cancer Res. 40: 3570-3575, 1980.

Twardzik, D. R., Reed, C. D., Weislow, O. S., and Fowler, A. K.: The effect of local anesthetics on cell surface receptors for the major envelope glycoprotein of murine leukemia virus. Int. J. Immunopharmacol. 2: 111-116, 1980.

Giron, D. J., Patterson, R. R., Hellman, A., and Fowler, A. K.: Enhancement of encephalomyocarditis virus replication in L cells treated with insulin Proc. Soc. Exp. Bio. Med. 166: 305-309, 1981.

Twardzik, D. R., Ranchalis, J. E., and Fowler. A. K.: Interaction of murine thymocyte histocompatibility antigens with envelope glycoproteins of Rauscher murine leukemia virus. In Lapin, Boris (Ed.), Russia Publishing Firm, U. S. S. R. (in press).

Weislow, O. S., Fisher, O. U., Twardzik, D. R., Hellman, A., and Fowler, A. K.: Depression of mitogenic-induced lymphocyte blastogenesis by baboon endogenous retrovirus associated components. Proc. Soc. Exp. Bio. Med. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04829-07 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

A Genetic Relationship Between a Novel Retrovirus and Intracisternal A Particle Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Callahan	Microbiologist	LCMB	NCI
Others:	Edward Kuff	Section Chief	LB	NCI
	Kira Lueders	Microbiologist	LB	NCI

COOPERATING UNITS (if any)

University of California, Medical School, San Francisco, California

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.6	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies were undertaken to elucidate the genetic relationship between the genome of a novel retrovirus (designated M432) isolated from M. cervicolor and the intracisternal type A particle (IAP) genome. The M432 retroviral genome was molecularly cloned in bacteriophage lambda and the nucleotide sequence of the long terminal repeat (LTR) of the M432 genome was determined. Comparative analysis of the M. musculus IAP and M432 retroviral genomes revealed two regions of sequence homology. The sequences which are unique to the M432 viral genome are not well conserved in species other than M. cervicolor, whereas, the IAP related sequences were shown to be highly conserved in all murine species.

Project Description

Background and Rationale: We previously reported the isolation of a novel class of endogenous retrovirus from the Asian species M. cervicolor and later M. caroli. By immunological and molecular hybridization criteria this class of retroviruses is unrelated to immune type B and type C retroviruses or retroviruses of other mammalian species. Recently Kuff et al. showed that the M432 genome did exhibit partial homology with the IAP genome of M. musculus. IAPs are retrovirus-like entities observed in large quantities budding from the endoplasmic reticulum into cisternae in plasma cell tumors and certain mammary tumors of the BALB/c strain of M. musculus. With the exception of early embryos or oocytes IAPs are infrequently observed in normal adult tissue. The IAP core is composed of a major internal protein P73, a reverse transcriptase and an RNA genome. There is no evidence for envelope proteins. In collaboration with E. Kuff, we showed that the major internal protein of these two types of particles is immunologically related, whereas, other M432 viral associate proteins did not react with the IAP P73.

Objectives: To determine at a molecular level the relationship between the M432 retroviral and IAP genomes and what role they play in certain murine tumors and early embryogenesis.

Major Findings: The M432 retroviral genome was molecularly cloned in bacteriophage lambda using linear unintegrated viral DNA from acutely infected tissue culture cells. The organization of the genome was determined by restriction endonuclease analysis. Like other members of retroviridae, the M432 viral genome contains a set of sequences repeated at each end of the genome and are designated the LTR. The nucleotide sequence of the M432 viral LTR has been determined and possesses the following characteristics: (a) it is 350 bp long; (b) the sequence overall is unrelated to the reported LTR sequences for mammalian and avian type C, murine type B retroviruses and M. musculus IAP LTRs, (c) short sequences are present which are similar, but not identical to recognition sites implicated in the regulation of RNA transcription and poly A addition to RNA.

The relationship between the M. musculus IAP and M. cervicolor M432 retroviral genomes was determined by heteroduplex and restriction endonuclease analysis. The 8.9 Kbp M432 retroviral and 7.0 Kbp IAP genomes contain a major region (3.8 Kbp) of sequence homology. This region begins respectively 3.3 Kbp and 1.5 Kbp from the 5' end of the M432 and IAP genomes. In terms of biological function the major homology region appears to begin near the end of the "gag" gene, spans the "pol" gene, and ends near the beginning of the "env" gene. A smaller region (0.6 Kbp) of the weak homology is observed immediately adjacent to the 3' LTRs of the respective genomes.

Restriction endonuclease analysis of cellular DNA from different species of Mus revealed that DNA sequences characteristic of the IAP genome are present in all mouse species tested whereas the viral sequences unique to the M432 genome are detected only in M. cervicolor and a closely related species M. cookii. Cellular DNA from M. caroli did not react with probes representing the unique portions of the M432 genome, although we have previously isolated a retrovirus which shares approximately 25% sequence homology with the M.

cervicolor M432 retroviral genome and a similar level of homology with M. cervicolor cellular DNA. We suggest that the observed homology between the two retroviral genomes may largely be due to their content of IAP related sequences which are common to both species.

In other studies we have examined normal adult tissues of M. cervicolor for the expression of M432 retroviral or IAP-related RNA sequences and proteins. Low, but detectable levels of RNA sequences partially related to the M432 viral genome were detected in adult spleen, kidney, and Peyer's patch tissues as well as lymphoma tumor tissue. Whether these sequences are IAP or viral specific is currently being tested. IAP were infrequently observed in electron micrographs of various tumors and adult tissues of M. cervicolor, whereas particles characteristic of the M432 retrovirus were not observed. In contrast, early embryos of M. cervicolor and M. pahari, like M. musculus each consistently contained large numbers of IAP.

Significance to Biomedical Research and Proposed Course: We tentatively conclude from these studies that the M432 virus exemplifies a class of retroviridae formed by recombination between an endogenous IAP genome and some as yet unidentified viral or newly evolved cellular sequence. An analogous phenomenon is known to have occurred under both experimental and natural conditions in the formation of mammalian and avian sarcoma virus genomes, except that infectious type C viruses were involved in these cases. Sequences related to the unique portions of the M432 virus were found in the cellular DNA of all individual M. cervicolor cervicolor and M. cervicolor popaeus mice that have been tested, suggesting that these sequences are endogenous to the cervicolor species. However, it is not yet known whether they are associated with IAP-related sequences in the cellular DNA or whether they form part of some other retroviral entity. Meanwhile, it is interesting to consider the possibility that IAP, although extracellularly non-infectious, may be capable of an intracellular "infection" with re-insertion of their genome at new sites in the cellular DNA or germline. It is relevant to this possibility that IAPs contain reverse transcriptase activity and have terminally redundant genomic sequences, elements which in other retroviridae are involved in intergration of the viral genome into cellular DNA. Thus the infectious M432 retrovirus could have arisen as a result of an insertion of the IAP genome into an existing retroviral gene or some other cellular DNA sequence peculiar to the M. cervicolor species. If this model is correct, an analogous event may have been involved in formation of the related retrovirus isolated from M. caroli; and similar chimeric extracellular viruses with incorporated IAP sequences may be anticipated for other mouse species. Similarly, a genetically mobile IAP genome could account for the large number of copies of IAP genes in M. musculus (1000/per haploid genome or 0.3% of the cell DNA) as compared to other species which may be relevant to the etiology of plasma cell and certain mammary tumors of BALB/c mice.

To further elucidate the nature of the relationship between the M432 retrovirus and the IAP genome, we are focusing our current efforts on a comparative analysis of molecular clones of the M. cervicolor popaeus IAP and M432-related DNA sequences. Cellular DNA from this species contains only 25 copies of IAP-related sequences, which should simplify this analysis. Furthermore, the existence of molecular clones of the IAP genome may facilitate the determination of their biological activity and transmissibility as it relates to their expression in mammary and plasma cell tumors as well as early embryos.

Publications:

Callahan, R., Kuff, E. L., Lueders, K. K., and Burkenmeier, E.: Structural and genetic relationships between an endogenous retrovirus (M432) of Mus cervicolor and intracisternal A particles of Mus musculus. In Fields, B., Jaenisch, R., and Fox, C. F. (Eds.): Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, Inc., 1980, pp. 197-206.

Callahan, R., Hood, M., Birkenmeier, E., and Mark, G.: The molecular cloning of the novel M. cervicolor popaeus endogenous retrovirus M432 and the sequence of its LTR. J. Virology (In Press).

Callahan, R., Lueders, K. K., Birkenmeier, E., and Kuff, E.: The genetic relationship between a novel M. cervicolor retrovirus (M432) and intracisternal type A particles of M. musculus. J. Virology (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04831-10 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on Reticulum Cell Neoplasm Type B in BALB/c Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.M. Merwin	Research Biologist	LCMB	NCI
Other:	R. Asofsky	Chief	LMI	NIAID

COOPERATING UNITS (if any)

George Klein and Francis Wiener, Karolinska Institutet, Sweden

LAB/BRANCH

Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were continued on over 50 murine lymphocytic neoplasms most of which have surface immunoglobulin (sIg) and, therefore, are probably of B rather than T lymphocytes. Some lines were maintained in transplant and some new ones were established to aid in correlating tumor characteristics with the sIg isotypes. The sites of tumor origin suggest that tumors are derived from cells in different stages of maturity, some from cells before antigen activation and some from cells after activation. Further observations have been made on interrelationships between sites of growth, lengths of latent periods, types of change with transplant and the isotype of the sIg. In some lines the incidence of tumor cells with sIg is different in the spleen than at the subcutaneous site, suggesting that the sIg may be extrinsically formed. The fact that almost all of these spontaneous lymphocytic neoplasms arose in either the Peyer's patches or in the mesenteric node, which is the main drainage node of the intestine, or in the spleen, which clears the blood of antigens including those antigens from the gut, suggests antigens originating in the gut are implicated in the induction of these tumors.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

To determine the relationships between the sIg isotypes and other tumor properties by analysis of data already obtained on transplant lines of over 50 lymphocytic neoplasms and by either continuing transplants of some old lines or establishing new lines with desired characteristics.

Major Findings:

Almost all tumors arose in either the gut-associated mesenteric node or Peyer's patches or in the spleen. The tumors originating in the mesenteric node or Peyer's patches include most with the S or G sIg isotype, about half of those with P and about one third of those with M. These lines could have originated from antigen-activated cells in the recirculating pool of lymphocytes, possibly stimulated to become neoplastic by antigens from the gut. Many tumors with sIg M or P arose in the spleen and the tumors with sIg M had characteristics indicating less mature cells not yet activated by antigen. Conceivably, antigens from the gut might reach splenic B lymphocytes through the blood stream and play a role in tumor induction without causing maturation to the antigen activated stage. Thus, antigens from the gut may have been implicated in the induction of all these tumors. Growth at extralymphative sites, such as growth at the site of subcutaneous inoculation, usually was present in the first generation of transplant, however, in several sIg M lines, this type of growth developed after a number of transplant generations. The change was associated with a long latent period in some and with a change in isotype in the two lines tested before and after the change. It was previously reported that this change seems to be independent of tumor antigenicity. In transplant lines, Peyer's patches, one of the sites invaded by cells of the recirculating pool of lymphocytes, are enlarged occasionally after long latent periods in lines of A or G sIg isotypes. A new line was established in which enlarged Peyer's patches developed in most transplant generations after short latent periods. This is the only line found with both IgA and IgG on the cell surface. In collaborative studies on the karyotype of 6 of these spontaneous tumors, G-banding showed that trisomy 15 was the dominant change in 5 of the 6 lines in early transplant generations. This observation suggests that nondisjunction of chromosome 15 may be associated with the genesis of B cell lymphomas and that chromosome 15 contains genes that may play an important role in the responsiveness of this type of cell to growth controls.

Significance to Biomedical Research and the Program of the Institute:

With each stage in maturation of B lymphocytes there are changes in surface phenotype, in migratory rates and in homing patterns relative to function. Tumor lines provide a large number of cells arrested at a certain stage of differentiation and as such can serve as experimental models of human B lymphocytic neoplasms. For example, tumors that changed from growth in the lymphatic tissues to growth mainly at extra lymphatic sites could be used as a model for experimental manipulation that can provide information on conditions causing extralymphatic growth of human tumors.

Proposed Course:

To prepare the results of these studies for publication.

Publications:

Wiener, F., Babonits, M., Spira, J., Bregula, U., Klein, G., Merwin, R., Asofsky, R., Lynes, M., and Haughton, G.: Chromosome 15 trisomy in spontaneous and carcinogen-induced murine lymphomas of B-cell origin. Int. J. Cancer. 27: 51-58, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04922-15 LCMB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunoprevention and Immunotherapy of Cancer in Experimental Animals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.J. Huebner	Head, Viral Immunology Section	LCMB	NCI
Other:	J.S. Rhim	Research Microbiologist	LCMB	NCI
	A. Bare	Bio. Lab. Tech.	LCMB	NCI
	R. Trimmer	Bio. Lab. Tech.	LCMB	NCI

COOPERATING UNITS (if any) S.A. Aaronson, D.V. Ablashi, R.H. Adamson and S. Sieber-Fabro, NCI; D. Fish and R.V. Gilden, FCRC; M.B. Gardner and S. Rasheed, USC; H. Lynch, Creighton U.; A. Krush, Johns Hopkins U.; E. Gardner and R. Moon, U. Utah; J. Coggins, U. Alabama; and C. Bowles, Hazleton Labs., Inc.

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION Viral Immunology Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Retroviruses that express viral and common tumor antigens prevented 3-MC cancers in mice and rats. 2) Retroviral immunotherapy of cancer(s) in dogs provided 8 long-term remissions and several possible cures. 3) Five retroviruses, concentrated 1000X, were safety tested in chimpanzees and demonstrated to be innocuous, thus suggesting the possibility for their use in humans immunotherapeutically. 4) Lymphoma epidemics in hamsters were traced to an ambient "viroid" present in contaminated rooms in studies at several laboratory sites (Drs. Huebner, Coggin and Gilden). 5) Retroviral therapy of carcinogen-induced liver tumor in primates has not been successful. However, immunoprevention experiments to suppress carcinogen-induced primate cancer appear feasible. 6) Skin transformation assays to identify individuals of cancer syndrome families at high risk for cancer was 80% successful in one test, 100% in the second. Forty specimens are now being run blindly in 3 laboratories for confirmation of results.

Project Description

Objectives:

1. Immunoprevention and immunotherapy approaches to cancer prevention and treatment in outbred species, including dogs, monkeys, chimpanzees and humans.

2. Development of simple and reproducible skin transformation assays for identification of individuals at high risk of cancer in families carrying autosomal dominant traits specific for cancer, and in persons heavily exposed to environmental or industrial carcinogens (i.e., smokers, asbestos workers).

Major Findings:

Prevention of cancer in the C57Bl/6 mouse. Immunization with the radiation leukemia virus (RadLV), the endogenous recombinant retrovirus of the C57BL/6 mouse, prevented 3-MC induction of cancer to a very significant extent, confirming previous observations by Whitmire and Huebner (1972). A variety of other recombinant retroviruses (GLV, RLV, AKR-247 and KiMSV) failed to protect against 3-MC-induced cancer.

Prevention of cancer in the NIH Swiss mouse. AKT-8, a mink cell line transformed by AKR virus, provided considerable protection against 3-MC-induced cancer in two experiments, as follows:

Experiment A	3-MC Tumors on Day	
	100	139
Immunized	0/12	2/12
Control	1/14	11/14
Significance (p value)	-	0.002

Experiment B			
	100	200	300
Immunized	1/24	7/24	8/24
Control	7/27	14/27	17/27
Significance (p value)	0.04	0.09	0.03

Prevention of 3-MC-induced tumors in the rat. Retroviral vaccines (.02 ml dosage of 1000X virus) were given intraperitoneally to 40 weanling Fischer rats 10 days prior to 3-MC challenge. The retroviruses used included RLV, AT124, RaLV (the endogenous rat retrovirus), KiMSV, RaSV and M55. Results are as follows:

		3-MC Tumors on Day		
	200	300	400	500
RaLV vaccine	1/40	1/40	10/40	20/40
AT-124 vaccine	2/40	8/40	15/40	20/40
Control	7/40	23/40	31/40	36/40
Significance (p-value)				
RaLV	0.03	0.0001	0.0001	0.0001
AT-124	0.08	0.0006	0.0003	0.0001

None of the other retroviruses (RLV, KiMSV, RaSV, M55) were protective against 3-MC-induced tumors.

The evidence in both the mouse and rat systems is that the preparations used which were successful provided the antigenic determinants necessary to suppress carcinogenesis. In the C57BL/6 and NIH Swiss mice, the RadLV, the endogenous retrovirus of the C57BL/6, and the AKT-8, a mink cell line transformed by the ARK, another endogenous mouse virus, contained the antigenic determinants necessary to prevent 3-MC tumor induction. In the rat, only the RaLV, the endogenous rat virus, and the AT-124, a mouse virus with shared determinants with the rat virus, were protective.

Retroviral immunotherapy of spontaneous cancer in dogs. In collaboration with Dr. C. Bowles and Dr. J. Stunkard, we achieved long-term remissions of primary and metastatic cancer, and possibly three cures, in eight dogs utilizing highly concentrated (1000X) naked retroviruses, alone, or in combination with, unconcentrated preparations administered intravenously and/or intraperitoneally.

In our initial attempt at therapy, a small female "Shitzu" carrying six walnut size lymphomas was inoculated intravenously with 3 ml of baboon virus. A mild anaphylactic response was suppressed with epinephrine. Five days later, the lymphomas appeared soft to the touch and swollen when palpated. At nine days, the lymphomas were not palpable. The dog, although still active, was euthanized at the request of the owners since she was going blind. A complete and exhaustive autopsy performed in the veterinary facilities at the NIH revealed no evidence of cancer at the sites of the lymphomas or in other tissues.

Current results. Although 40 dogs were included in our treatment panel, many were cachetic and unable to respond. The dogs which responded, as reported above, were those evaluated by their veterinarians to be in "fair" or "good" general physical condition. Eight dogs are currently in long-term remission. Three of these, given multiple treatments over the course of several months, now appear to be free of demonstrable cancer or symptoms. These are being followed closely, and will be given prophylactic "boosters" to ensure their continued health. The eight dogs in remission were diagnosed clinically and pathologically as follows:

1 Metastatic adenocarcinoma of endocrine origin (probably thyroid)
1 Hemangiopericytoma
1 Adenocarcinoma (uterus - inoperable)
1 Metastatic lung tumor
2 Testicular carcinomas
1 Melanoma
1 Squamous cell carcinoma
1 Hemangiosarcoma*

*This dog died at the age of 13 years, 11 months after the last treatment. At autopsy, there was evidence of residual cancer, suggesting that follow-up treatments would have been advisable.

The mechanism responsible for the rejections is attributed to a powerful cell-mediated immunity reacting to the preferential growth of these viruses in tumor cells, thus, triggering the immunological reaction. Biopsies of residual tumor tissue in treated dogs were found to have numerous viral particles.

Most of the past year has been spent in determining the most efficacious and safe treatment regimens. Prior chemotherapy, with or without steroids, was not advantageous; however, surgical debulking of tumor contributed significantly to the effectiveness of retroviral treatment. We also found that the intraperitoneal route of administration was as effective as the intravenous route and minimized the threat of anaphylaxis which we experienced when using the intravenous route.

Safety tests of retroviruses used in prevention and/or therapy. On the advice of the Bureau of Biologics, five of the commonly used retroviruses concentrated at least 1000X, with titers of 10^4 - 10^6 in the S^+L^- mink cell focus-forming assay, were tested in two adult chimpanzees. Five ml of each of the following retroviruses were inoculated intravenously in consecutive order: M7 (baboon), RD114, C57L, M55 and 1504A. The chimpanzees, which had been lightly tranquilized, demonstrated no stress or untoward reactions during or after treatment or in the months following. These results would suggest that retroviral immunotherapy of cancer in humans would be equally without risk of anaphylaxis or other hazard.

Lymphoma epidemics in hamsters. In collaboration with Drs. Joseph Coggin and Raymond Gilden, studies on a filterable, ambient oncogenic agent derived from horizontally transmitted lymphomas in Syrian hamsters were continued after a hiatus of several years. The agent was active in culture preparations, yet could not be visualized in the electron microscope. It could be inactivated with DNAse, but not by RNAse or trypsin. Although we have not been able to detect or identify the agent, it was capable of producing recurrent epidemics of lymphoma in several facilities, including laboratories at Oak Ridge (where Dr. Coggin first discovered the disease), the University of Alabama, and here at the NIH, even after exhaustive disinfection between epidemics. Studies are continuing to determine whether the agent may represent the DNA of the hamster wart virus, a virus which is known to replicate spontaneuously in hamsters and which has been associated with oncogenesis in hamsters. Other DNA viruses are also under investigation.

Immunotherapy of chemically induced cancers in nonhuman primates. In collaboration with Drs. R. H. Adamson and S. Sieber-Fabro, one cynomolgus and two rhesus monkeys bearing MNNG-induced liver tumors have been treated with retroviruses, with no apparent effect to date. Only two treatments have been administered to the cynomolgus and one each to the rhesus because of the monkeys' generally frail condition. This will be followed up more intensively in the coming year. We hope to embark on a cancer prevention program with Drs. Adamson and Sieber-Fabro in future studies to try to prevent tumor induction by prior treatment with retroviruses.

Skin transformation assays for detection of people at high risk for cancer. In collaboration with Drs. Henry Lynch and Murray Gardner and Mrs. Anne Krush, we obtained biopsies from individuals of families carrying autosomal dominant genes for high (100%) cancer risk (Gardner's, FAMMM and ACR syndromes), in accordance with recommendations made at a workshop on "Assays for Identification of High Risk Individuals in Autosomal Dominant Gene Cancer Family Members," held January 29-30, 1980. Biopsies were cultured to 3-5 passages and then infected with serial log doses of either KiMSV or KiMSV(baboon). Cells derived from individuals at high risk transformed at virus titers of 10^2-10^3, whereas cells at normal risk required 10^4 or higher titers to transform. The results correlated well with specific markers in Gardner's syndrome patients. Results were later confirmed in blind tests as follows: Of the initial 12 specimens, the KiMSV assay was at least 80% accurate in identifying individuals known to be at high risk by virtue of other markers. Two positives without physical markers were children, who may or may not have the trait. A second test of 8 specimens, run under the same conditions, was virtually 100% accurate. An additional 40 specimens have been sent blind by Dr. Eldon Gardner, from his repository of cancer families, and are being tested by three laboratories, utilizing at least two separate assays. Results are not in as yet. After completion by all laboratories, the code will be broken by Dr. E. Gardner. Results should be available within the next year.

Rabbit studies. In collaboration with Dr. Ablashi, rabbits bearing lymphomas induced by herpesvirus saimiri (HVS) will be treated immunotherapeutically with retroviruses. These studies have not proceeded beyond the induction stage, pending information on which retroviruses grow well in rabbit cells. There is some question about the probable efficacy of retroviruses in this system since the retroviruses used to treat such virus-induced cancers may augment HVS oncogenicity rather than trigger a host immunological response against the HVS-induced tumors.

Significance to Biomedical Research and the Program of the Institute: The study of retroviruses as cause and prevention of cancer in lower animals has provided insight concerning mechanisms of carcinogenesis which, in turn, has provided the rationale for appropriate intervention. Immunoprevention of spontaneous and carcinogen-induced cancers of lower mammals demonstrates the feasibility of developing a vaccine for use in persons at high risk for cancer by virtue of genetic susceptibility or environmental exposure (i.e., heavy smokers). Retroviral immunotherapy of spontaneous and induced cancers in dogs and experimental rodents and subhuman primates has proved innocuous in all systems, and is effective against spontaneous cancers of all types in dogs. Protocols are now on test to determine the optimal treatment regimens to adopt.

Prototypes of the retroviruses used in vaccines and immunotherapy, concentrated 1000X, were consecutively inoculated intravenously into chimpanzees and found to be harmless. These safety tests were performed on advice of the Bureau of Biologics and have demonstrated the feasibility of eventually using retroviruses in prevention and therapy of human cancer.

A skin transformation assay was developed by Drs. Huebner, Rhim, M. Gardner and S. Rasheed to identify individuals at high risk for cancer prior to onset of symptoms. This assay, in combination with a prophylactic cancer prevention program, including, possibly, the use of retroviral vaccines, may eventually provide the best approach to cancer control.

Identification of the source of an oncogenic "viroid," suspected to be the DNA product of the hamster wart virus, or perhaps a DNA product of an activated oncogene of the hamster, would provide insight into a naturally occurring, very puzzling series of reproducible hamster lymphoma epidemics.

Proposed Course:

The mouse and rat cancer prevention studies are complete. The approaches developed in these systems should eventually be of value as models for prevention of cancer in high risk humans. The retroviruses which are proving efficacious against spontaneous cancer in the dog have been demonstrated to be safe in protocols suggested by the Bureau of Biologics.

Immunotherapeutic trials of a variety of retroviruses will continue in dogs and primates, varying protocols to determine the most effective regimens. This represents the section's highest priority effort.

With Dr. J. Coggin, collaborative studies on the hamster "viroid" responsible for lymphoma epidemics will continue on a modest scale. Most of the work in the future will probably be done at the molecular level, although *in vivo* experiments are now being repeated on a small scale for additional information and to provide materials needed for continuing studies.

Blind tests of 40 skin biopsies derived from members of "cancer families" and normal controls will be completed during the coming year. In collaboration with Dr. Jack Frankel, application of the test on a larger scale will also be undertaken in groups presumed to be at high risk of cancer by virtue of environmental exposure (smokers, asbestos workers, etc.). The latter will be initiated after confirmation of test validity in small trials within such populations. If parameters can be established to exclude false positives or negatives, the test would provide inexpensive and feasible assays for use by state health and other medical laboratories.

Publications:

Rhim, J. S., Huebner, R. J., Arnstein, P., and Kopelovich, L.: Chemical transformation of cultured human skin fibroblasts derived from individuals with hereditary adenomatosis of the colon and rectum. Int. J. Cancer 26: 565-569, 1980.

Coggin, J. H., Oakes, J. E., Huebner, R. J., and Gilden, R. V.: Isolation of an unusual filterable oncogenic agent from horizontally transmitted Syrian hamster lymphomas. Nature 290: 336-338, 1981.

Fish, D. C., Demarais, J. T., Djurickovic, D. B., and Huebner, R. J.: Prevention of 3-methylcholanthrene-induced fibrosarcomas in rats pre-inoculated with endogenous rat retrovirus (a brief report). Proc. Nat. Acad. Sci. 78: 2526-2527, 1981.

Rhim, J. S., Trimmer, R., Arnstein, P., and Huebner, R. J.: Neoplastic transformation of chimpanzee cells induced by adenovirus type 12-simian virus 40 hybrid virus. Proc. Nat. Acad. Sci. 78: 313-317, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04925-10 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of RNA Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Sarma	Research Microbiologist	LCMB	NCI
Other:	P. Reddy	Visiting Scientist	LCMB	NCI
	J. Dahlberg	Research Microbiologist	LCMB	NCI
	S.A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS (if any)

M. Sponavgle, Maryland Wildlife Center, Hagerstown, Maryland

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION
Animal Virology and Field Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were undertaken to determine the presence of retroviruses detectable by cell co-cultivation and virus induction methods in fibroblastic cultures of wild bats and kidney epithelial cultures of a woodchuck. Attempts were made to recombine (rescue) cellular 'sarc' genes of normal cat fibroblastic cells with feline leukemia virus by induction with a halogenated pyrimidine, 5-iododeoxyuridine. Studies were undertaken to molecularly clone the SM strain of feline sarcoma virus.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

This laboratory is concerned with studies of the biology, prevalence, transmission, expression, rescue and characterization of selected RNA tumor viruses and their genomes in natural and experimental model systems by methods involving techniques of virology, cell biology, immunology and molecular biology.

Major Findings:

The wild rodent woodchuck has been a valuable animal model in studying the human hepatitis B virus inasmuch as a virus similar to the human hepatitis B virus causes a similar hepatic disease in woodchucks. Recent molecular hybridization studies have shown the presence of human hepatitis viral DNA in integrated form in primary liver carcinomas of patients who had suffered from hepatitis B infection. In a preliminary study to establish whether or not endogenous type C RNA tumor virus(es) occur in woodchucks and whether they play a primary or co-carcinogenic role in hepatic cancers of woodchucks, an attempt was made to determine the presence of such a virus demonstrable by virus induction techniques. A cell line of kidney epithelial cultures was established from an adult male woodchuck obtained through the courtesy and assistance of the Maryland Wildlife Center, Hagerstown, Maryland. At various *in vitro* passage levels, the cells were treated with IUDR (30 μg/ml for 48 hours) and then separately co-cultivated with human rhabdomyosarcoma cells (RD) and cat cells. The release into the culture medium of a retrovirus was determined by reverse transcriptase assays. A virus detectable by this procedure was not found even after 4 months of continuous cell co-cultivations with RD cells or cat cells.

Virus induction studies to determine presence of endogenous retrovirus in bats.

Three young male bats were obtained from a student trapper in Frostburg, Maryland. Mixed cultures of testicles and kidneys were prepared and maintained over several passages *in vitro*. These cultures were tested for the presence of endogenous retrovirus by induction with IUDR (30 μg/ml for 48 hours), followed by cell co-cultivation with RD cells and cat cells. Prolonged separate cell co-cultivations over a 3 month period failed to demonstrate the presence of endogenous retrovirus as determined by assays of clarified culture fluids for reverse transcriptase activity.

Attempts to rescue cellular sarc genes with a leukemia virus.

Sarcoma viruses arise in nature by a recombinational event between leukemia viral genes and normal cellular sarc genes with the deletion of some leukemia viral sequences such as the *env* gene. We observed that feline cells chronically infected with feline leukemia virus (FeLV) and maintained by serial passage over several months, frequently tended to show morphologic evidence of cell transformation followed by reversion to normal appearance. Attempts to isolate a cell transforming virus from the infected cat cultures at the time of morphologic transformation met with little or no success. In one experiment, such a virus capable of transforming cat cultures was recovered, but in low titer and was lost in subsequent passages.

In an *in vitro* attempt to recombine cellular sarc sequences in normal cat cells with FeLV sequences, cultures of cat embryo fibroblasts were infected with FeLV A or FeLV B. Chronically infected, but normal appearing cultures were thus established. They released high titers of virus as determined by reverse transcriptase assays. The infected and control cultures were treated with IUDR (30 µg/ml for 48 hours). It was found that the FeLV-infected cat cultures, which were between 32nd and 40th passages *in vitro*, responded to IUDR treatment by a transient change in cellular morphology mimicking cellular transformation. This morphologic change lasted for 7 to 14 days following treatment with IUDR.

Such a morphologic change was not observed in parallel control cat cultures not infected with FeLV, but which were similarly treated in parallel with IUDR. Attempts to isolate a cell transforming virus from IdU-treated FeLV-producing cultures were not successful. In one experiment, a 'fixation' of the cell transformation event was attempted as follows: Immediately after IdU treatment, IdU-treated and control cat cultures were co-cultivated with normal cat cells, in equal parts, to allow the infection of normal cells by a putative sarcoma virus released by FeLV infected cultures which were exposed to IdU. After 4 days of co-cultivation, the cells were then planted in a soft agar (0.3%) containing medium and maintained for 4 weeks. Several colonies of cell growth were observed in FeLV-infected cat cells that had been exposed to IdU, but not in parallel control cultures consisting of cat cells exposed to IdU or FeLV-infected cat cells or normal cat cells. Transformed cultures were established from 7 colonies, but all cultures were lost due to fungal contamination attributable to renovation of surrounding areas now in progress.

SM strain of feline sarcoma virus: molecular cloning studies.

Studies are under way to clone the integrated DNA of the SM strain of feline sarcoma virus in BALB/c nonproducer transformed culture (S. Aaronson). High molecular weight DNA was prepared of the SM-FeSV BALB/c cells and control BALB/c 3T3 cells. Preliminary restriction mapping studies have shown that 2 of 6 restriction enzymes that have been tested, Bam HI and Xho I, cut into the viral genome as determined by molecular hybridization performed with restricted DNA transferred onto nitrocellulose membranes by the Southern blotting technique. The probe used was nick-translated, cloned ST strain of feline sarcoma virus. Cloned subgroup B FeLV, obtained from Dr. J. Mullins (California Institute of Technology, Pasadena), will be used as probe in future studies. Transfection experiments are under way to determine the suitability of the 6 restriction enzymes in cloning the SM strain as determined by the ability of the restricted fragments to transform BALB/c cells.

Significance to Biomedical Research and the Program of the Institute:

Though endogenous retroviruses, especially type C RNA viruses, are presumed to be present in covert form in most, if not all, vertebrates, limited experiments described herein provided some information on the inability to demonstrate such viruses in woodchucks and bats by the methods used. Experiments to produce a cell transforming virus in vitro by a recombination between cellular sarc sequences and leukemia viral sequences, though unfruitful for the most part, do suggest the need for additional experiments using a variety of mutagenic agents and soft agar techniques to maximize the chances of recovering a recombinant in an infectious form. Such cell transforming sarcomagenic recombinants that arise spontaneously in vivo could thus be conceivably produced in vitro under defined experimental conditions. Cloning of the SM-FeSV should enable us to understand the origin, biology and interrelationships between this virus and other feline sarcoma viruses and their gene products, including those involved in the cell transformation process.

Proposed Course:

Molecular cloning studies of the SM-strain of feline sarcoma virus will be continued. Collaborative studies are now being initiated with Dr. J. Dahlberg to fully characterize the woodchuck kidney epithelial cell line and its putative endogenous retroviruses.

Publictions:

Gardner, M.B., Rongey, R., Sarma, P., and Arnstein, P.: Search for retrovirus particles in spontaneous tumors of parakeets. Verterinary Pathology. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04930-10 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biology of Natural and Induced Neoplasias

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Arnstein	Veterinary Director	LCMB	NCI
Other:	R. Huebner	Head, Viral Immunology Section	LCMB	NCI
	S. Aaronson	Chief	LCMB	NCI
	J. Rhim	Research Microbiologist	LCMB	NCI

COOPERATING UNITS (if any) C. Graham, Internat. Cent. Env. Safety, Holloman AFB, NM; A. Hackett, Peralta Cancer Inst.; R. Gilden and D. Fish, FCRC; R.W. Emmons, Calif. Dept. Health Services; M. Gardner, USC; J. Levy, U. Calif.; W. Nelson-Rees, Naval Bio. Res. Lab., Oakland

LAB/BRANCH

Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Chimpanzee and human cultures have been permanently altered from normal to preneoplastic using hybrid Ad12-SV40 tumor virus. In one instance normal chimpanzee skin has been successfully transformed to an established malignant chimpanzee line (sarcoma).

2. Mouse leukemia retrovirus recombinants between the "experimental" Rauscher and the "endogenous" xenotropic strains behave as the fully virulent leukemogenic Rauscher parent in immunodeficient hosts, but are much less pathogenic or non-pathogenic in immunocompetent siblings.

3. Human and animal xenografts on athymic nude mice have been used to study tumor progression and malignant transformation.

4. Psoralen-UV photochemical inactivation of tumor viruses before infecting cells has produced less cytopathic viral transformation.

PHS-6040
(Rev. 2-81)

Project Description

Objective:

1. Develop methods which consistently induce malignant transformation in normal primate cells; subsequently produce vaccines and perform vaccine trials in primates and lower species. Ultimately, these approaches may be used in human cancer vaccine development.

2. Determine in vivo biological activity (including induction of malignancy) by genetically fully mapped mouse leukemia viruses in their natural hosts (newborn mice). These viruses, genetically "engineered" in Dr. Aaronson's laboratory, will pinpoint genomic loci for specific neoplasias.

3. Study transplanted human and selected animal xenografts for malignant behavior, tumor-specific antigen production, malignancy-related genes or possible new viruses.

4. Conduct studies of in vitro and in vivo photochemical effects on normal and neoplastic tissues and tumor viruses, using various psoralens and long wave UV light.

Major Findings:

1. The studies of the effect of tumor viruses and chemical cocarcinogens on normal human and chimpanzee cells have produced additional evidence that such cultures from the genera Pan and Homo are very similar in their responses. Human and chimpanzee cultures (either adult skin or fetal fibroblasts) from 14 different individuals were infected with Ad12-SV40 hybrid virus (highly oncogenic in hamsters) with or without the co-carcinogenic action of tetradecanoyl phorbolacetate (TPA). In each case a profound permanent morphologic change was produced, best described as "preneoplastic" and possessing some of the requisites for the malignant transformation, i.e., accelerated growth, loss of contact inhibition, loss of anchorage dependence, and delay or complete elimination of senescence (established "immortal" cell line production).

With one exception, these altered lines failed to produce malignant xenografts in athymic nude mice. The one exception is chimpanzee skin fibroblast from the donor Wes, which following Ad12-SV40 infection, agar cloning and selection of T-antigen positive but virus negative subline, produced invasive, serially transplantable sarcomas in the nude mice and could be readily reestablished from the mouse-borne tumors as karyologically "pure" but aneuploid Pan troglodytes (chimpanzee) karyotype (see Rhim et al.).

More recently, we found that a belated tumorigenic effect occurs in "nude" mice, provided they survive at least 6 months following engraftment of Ad12-SV40-altered (preneoplastic) human and chimpanzee lines, producing viable virions. At about 6 months, and at the exact location where the cells were injected, these mice suddenly develop rapidly growing sarcomas consisting of murine cells (i.e., de novo tumor induction as opposed to xenograft). Since the mouse and its tissues are supposedly nonpermissive for direct Ad12-SV40 viral infection, this phenomenon needs careful study.

2. Murine type C leukemia virus recombinants, which contain known segments of the proven virulent, Rauscher leukemia genome, and accurately defined genomic portions of the endogenous murine type C xenotrope (the latter is a ubiquitous murine retrovirus) have been isolated. The tumorigenicity of the various recombinants has been compared with that of prototype clones of classic leukemia viruses (Rauscher and Moloney). We have found that viruses with some xenotropic gene sequences inoculated into "euthymic" (normal) mice always result in either greatly delayed onset of lymphomas or complete absence of R-E neoplasia. (The strains used, 129J and NIH Swiss, are highly susceptible to the intact Rauscher and Moloney viruses). However, immunodeficient athymic nude litter mates of the above described mice succumb to leukemia.

No sparing effect for normal vs. athymic animals can be shown with unaltered Rauscher or Moloney leukemias, even with diluted virus. It thus appears that recipients of recombinant retroviruses tested mount a more successful anti-tumor defense than do those receiving intact (parent) Rauscher virus, provided the host is immunocompetent.

Host strain differences have also shown up with some viruses, although they are less profound, e.g., the 129J shows a definite delay in onset of Rauscher leukemia morbidity and mortality over the NIH Swiss (but ultimately all succumb).

3. Xenotransplantation experiments with human normal and neoplastic tissues have continued to contribute new information on the behavior of normal, preneoplastic and clearly malignant cultures in the immunodeficient animal host (athymic nude mice).

4. The photochemical inactivation (psoralen + long wave UV light) described in last year's report is being applied to the retrovirus and Ad12-SV40 hybrid virus carcinogenesis studies. One practical benefit, we found, is that the latter (Ad12-SV40), which ordinarily produced much cytopathology and "dirty" looking cultures due to cell debris before the onset of transformation, can be rendered much less cytotoxic without affecting its transforming potency by sublethal psoralen + light treatment.

Significance to Biomedical Research and the Program of the Institute:

1. Studies on transformation, carcinogenesis, reversion and xenografting primate tissue will be of great help in human cancer immunotherapy, prevention and research.

2. Understanding the pathogenesis of malignancy-specific retrovirus genes in rodents should contribute to the search for similar phenomena in human tissues.

3. The behavior of xenografts is an economical method of studying human cancers on a relatively economical (mouse) host, as well as distinguishing non-malignant from malignant among doubtful cultures.

4. Photochemical attenuation of tumor cells, viruses and normal tissue may offer leads towards cancer immunotherapy and aid in viral carcinogenesis procedures.

Publications:

Rhim, J. S., Huebner, R. J., Arnstein, P., and Kopelvich, L.: Chemical transformation of cultured human skin fibroblasts devised from individuals with hereditary adenomatosis of the colon and rectum. Int. J. Cancer 26: 565-569, 1980.

Rhim, J. S., Trimmer, R., Arnstein, P., and Huebner, R. J.: Neoplastic transformation of chimpanzee cells induced by adenovirus type 12-simian virus 40 hybrid virus. Proc. Natl. Acad. Sci. 78: 313-317, 1981.

Rhim, J. S., Arnstein, P., and Huebner, R. J.: Chemical transformation of cultured skin fibroblasts from humans genetically predisposed to cancer. In: Proceedings of the Fourth International Symposium on Prevention and Detection of Cancer. New York and Basel, Marcel Dekker, 1981 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04940-14 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Viruses in Experimental Oncogenesis and Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.A. Aaronson	Chief	LCMB	NCI
Other:	S.R. Tronick	Microbiologist	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI
	S. Devare	Visiting Associate	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	K.C. Robbins	Expert	LCMB	NCI
	P. Andersen	Senior Staff Fellow	LCMB	NCI
	J. Pierce	Staff Fellow	LCMB	NCI
	T. Storch	Research Associate	LCMB	NCI
	B. Weissman	Chemist	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
	K. Nagao	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)
R.J. Huebner, LCMB, NCI; R. Gallo, LTCB, NCI; E. Scolnick, LTVG, NCI; D. Bolognesi, Duke Univ.; R.V. Gilden, FCRC; J. Greenberger, Harvard Univ.; E. Hunter, Univ. Alabama; J. Merregaert, SCK, Belgium

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to elucidate the mechanisms of action of tumor viruses and to search for evidence of tumor viruses in humans with the eventual aim of developing rational approaches to prevention of human cancer. Topics of present interest include: 1) the biology of endogenous retroviruses, 2) the molecular biology of retrovirus replication and transformation, and 3) the application of knowledge gained from these studies to the search for tumor viruses of humans.

Project Description

Objectives:

1. To study the mechanisms of action of RNA and DNA containing tumor viruses.

2. To apply knowledge gained from experimental systems to search for a viral etiology to human neoplasia and to develop rational approaches to prevention and treatment of human tumors.

Methods Employed:

Standard and developmental techniques in virology, cell biology, immunology and biochemistry.

Major Findings:

1. Moloney murine sarcoma virus strain 124 (MSV-124) possesses the ability to transform cells but requires a type C RNA virus as a helper virus for its propagation as an infectious virus. We inquired whether natural selection during virus replication and transmission would lead to alteration of MSV-124 genetic information. Individual viruses were cloned in nonproducer transformants from MSV-124 stock that had undergone a single or multiple cycles of infection. Analysis of the ability of individual MSV-124 transformants to express helper viral gag gene products indicated the selection of variants that had lost the capacity to express some or all such MSV-124 coded information. The genomic size of individual MSV-124 variants was determined by electrophoretic and hybridization analysis of unintegrated proviral DNA. Viruses isolated following multiple cycles of infection were shown to contain deletions of up to 35% of the parental MSV-124 genome. Moreover, there was a striking natural selection for such mutants. Thus, the evidence indicates that MSV-124 deletion mutants possessed a significant growth advantage relative to the parental MSV-124 genome.

2. The sequence of the transforming region of the Moloney murine sarcoma virus genome has been determined by using molecularly cloned viral DNA. This region, 3.6 to 5.8 kilobase pairs from the left end of the molecule, contains the entire cellular insertion (src) sequence as well as helper viral sequences including the large terminal repeat (LTR). On the viral RNA strand, a long (1224 bases) open reading frame commenced to the left of the src-helper virus junction and terminated at a point 58 nucleotides into helper viral sequences to the right of src. Possible promoter and acceptor splice signals were detected in helper viral sequences upstream from this open reading frame. On the antiviral RNA strand, several promoter-like sequences, including one within the src region itself, were identified. However, no open reading frame downstream from these promoters was

detected in the antiviral RNA strand. The LTR was found to contain promoter-like sequences, as well as mRNA capping and polyadenylation signals. In addition, it possessed an 11-base inverted terminal repeat at each end. Thus, the structure of the Moloney murine sarcoma virus genome with an LTR at each end resembles that of prokaryotic transposable elements.

3. *In vitro* translation of virion RNA of Moloney murine sarcoma virus (MSV) *strain* 124 yielded major products having molecular weights of 63,000 (63K), 43K, 40K, 31K, and 24K daltons. A molecularly cloned subgenomic fragment of Moloney MSV comprised of the cellular insertion (*src*) region was utilized in hybridization arrest translation as a means of identifying products of the MSV *src* gene. MSV *src* DNA specifically inhibited synthesis of the 43K, 40K, 31K, and 24K proteins, implying that each of these proteins originated from within the MSV *src* gene. The MSV *src*-specific nature of this family of proteins was further confirmed by partial purification of MSV *src*-containing RNAs from MSV nonproducer cells. *In vitro* translation of enriched cellular RNAs yielded products with molecular weights identical to those of the 43K family of proteins synthesized from virion RNA. Nucleotide sequence analysis of the MSV transforming region has revealed a long open reading frame which includes five methionine codons (Reddy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 77: 5234-5238, 1980). The molecular weights of the four largest proteins that could be synthesized within this open reading frame corresponded closely to the molecular weights of the 43K family of proteins. Partial cyanogen bromide cleavage of each of the three largest proteins resulted in an uncleaved fragment having a molecular weight equal to that of the smallest (24K) protein. These findings provide direct biochemical evidence that the 43K, 40K, 31K, and 24K proteins are related in their carboxy-terminal regions, as well as information concerning the MSV *src* gene coding sequences from which each protein originates.

4. The integrated proviral genome of Abelson murine leukemia virus (A-MuLV) was cloned in λgt WES.λB bacteriophage following Eco RI digestion and enrichment of proviral sequences by sequential RPC-5 column chromatography and agarose gel electrophoresis. Recombinant DNA clones containing a 7.8 kbp Eco RI insert were shown to have the entire integrated Abelson-MuLV genome with both 5' and 3' ends flanked by mink cellular DNA sequences. This DNA fragment was shown to induce focus transformation upon transfection of NIH/3T3 cells. Moreover, focus-forming virus could be rescued from transformed nonproducer cells upon superinfection with a type C helper virus. A polyprotein of MW 120,000 (p120) containing MuLV *gag* gene determinants was invariably detected by immunoprecipitation analysis of individual transformants induced by the 7.8 kbp DNA. Molecularly cloned, integrated A-MuLV in its infectious form should be of use in elucidating the mechanisms involved in transformation by this virus.

5. The integrated form of simian sarcoma virus (SSV) was molecularly cloned in the Charon 16A strain of bacteriophage λ. By transfection analysis, the recombinant viral DNAs demonstrated the ability to transform cells in tissue culture at high efficiency. Such transformants possessed typical SSV morphology, expressed simian sarcoma associated virus (SSAV) gag gene products in the absence of virus release, and released SSV following superinfection with a type C helper virus. A physical map of the 5.8 kbp recombinant viral DNA clone, deduced from restriction endonuclease analysis, revealed a 5.1 kbp SSV genome containing 0.55 kbp long terminal repeats (LTRs) flanked by 0.45 and 0.25 kbp of contiguous host cell sequences. By R-loop analysis, the viral DNA molecule contained two regions of homology to SSAV, separated by a 1.0 kbp nonhomologous region. This SSV specific sequence was shown to be uniquely represented within the normal cellular DNA of diverse mammalian species, including human. Our results demonstrate that this primate transforming retrovirus arose in nature by recombination of a type C helper virus and a host cellular gene.

6. Recombinant viruses were generated in tissue culture between Rauscher-murine leukemia virus (MuLV) temperature-sensitive (ts) mutants restricted at different steps in virus replication and a mouse endogenous xenotropic virus, BALB:virus-2. Mutants utilized included ts 28, a late mutant which releases noninfectious viruses at 39°, and ts 29, a double mutant with a ts lesion in its reverse transcriptase and a late block affecting virus budding. Immunological typing of the translational products of clonal recombinant viruses made it possible to establish their partial genetic maps and localize regions of the viral genome affected by different ts lesions. Recombinants involving Rauscher-MuLV ts 28, invariably contained BALB:virus-2 p15, p12 and p30 proteins, localizing the late defect in replication by this mutant to the 5' moiety of the viral gag gene. All ts 29-derived recombinants contained the entire BALB: virus-2 gag and pol genes. Substitution of the pol gene is in agreement with the reported thermolability of Rauscher-MuLV ts 29 reverse transcriptase (Tronick et al., J. Virol. 16: 1476-1482, 1975). Substitution of the gag gene suggests that internal structural proteins are actively involved in the virus budding process. Rauscher-MuLV recombinants were utilized to establish the genetic map of the Rauscher-MuLV genome by oligonucleotide T_1 fingerprinting analysis. Detection of Rauscher-MuLV T_1-oligonucleotides in representative recombinant viruses, whose protein phenotypes were established by immunologic techniques, permitted their assignment to specific regions of the viral genome. The genetic map of Rauscher-MuLV generated in these studies should be useful in the identification and characterization of the viral gene(s) involved in leukemogenesis.

7. The major core protein (p28) of MMC-1, an endogenous type C virus of rhesus monkey (Macaca mulatta), was purified and subjected to structural and immunological analysis. The NH_2-terminal amino acid sequence of MMC-1 p28 showed extensive homology to the sequences of the major structural proteins (p30) of known mammalian type C viruses. Similarly, interspecies antigenic determinants shared by all the above viral proteins were detected in MMC-1 p28. Competition radioimmunoassays, together with the results of statistical analysis of the primary structure data, provided evidence that MMC-1 p28 is not more closely related to primate type C viruses of the Papio genus than to those isolated from rodents, cats or New World monkeys. MMC-1 p28 was found to be closely related structurally to the p30 protein of the avian reticuloendotheliosis virus (REV-A), a horizontally transmitted type C virus of putative mammalian origin. In addition, MMC-1 p28 and REV-A p30 shared a specific subset of antigenic determinants not present in any of the other avian or mammalian type C viruses studied. These findings suggest that MMC-1 and REV may have a common evolutionary origin.

8. Nonadherent tissue culture cell lines were established from normal bone marrow of a variety of mouse strains. The lines possessed morphological and histochemical markers of the basophil/mast cell and contained committed stem cells for metachromatic cells. Their derivation from normal marrow and their lack of tumorigenicity despite long-term culture makes these cell lines potentially important for studies of the mechanisms of allergic reactions and inflammation as well as the differentiation pathways involving this subset of hematopoietic cells.

9. "Pregnancy-specific" beta-1 glycoprotein (SP_1) was produced in vitro by 9 of 32 established cell lines derived from human malignant neoplasms. The 9 positive lines comprised a variety of cell types and SP_1 production ranged from 0.36-35.5 pmol/mg cell protein at confluence. The highest production was by a fibrosarcoma line. Sp_1 in medium and cells from this line were indistinguishable immunochemically and gel chromatographically from purified (Bohn) placental SP_1. None of the SP_1-positive lines produced placental lactogen; only 4 produced the beta subunit of CG. SP_1-positive lines that produced CG-α did not produce CG-β. Secretion of SP_1 and of CG-β by the fibrosarcoma line followed different time courses.

Significance to Biomedical Research and the Program of the Institute:

The systems that are being intensively investigated have provided a much better understanding of the biology and biochemistry of viral transformation. It is felt that a clear understanding of these phenomena will significantly speed progress in the search for a viral etiology of human cancer as well as approaches toward cancer prevention.

Proposed Course:

To continue research already in progress in the following major areas:
(1) mechanisms of action of mammalian sarcoma and leukemia viruses;
(2) induction and regulation of retroviruses in virus-negative cells;
(3) determination of the role of viruses in human neoplasia; and
(4) develop new research areas in model systems that pertain to human disease.

Publications:

Barbacid, M., Krakower, J., and Aaronson, S.A.: The search for evidence of humoral immunity to oncoviruses in man. In Todaro, G.J., and Essex, M. (Eds.): Viruses in Naturally Occurring Cancers. New York, Cold Spring Harbor Laboratory Press, 1980, pp. 869-883.

Canaani, E., and Aaronson, S.A.: Isolation and characterization of naturally occurring deletion mutants of Moloney murine sarcoma virus. Virology, 105: 456-466, 1980.

Canaani, E., Tronick, S., Robbins, K.C., Andersen, P.R., Dunn, C.Y., and Aaronson, S.A.: Cellular origin of the transforming gene of Moloney murine sarcoma virus. Cold Spring Harbor Symp. Quant. Biol., 44: 727-734, 1980.

Gardner, M.B., Barbacid, M., Rasheed, S., Grant, C., and Aaronson, S.A.: Humoral immunity in natural FeLV-exposed and experimental FeSV-inoculated house cats. In Hardy, W.D., Essex, M., and McClelland, A.J. (Eds.): Feline Leukemia Virus. New York, Elsevier/North Holland Press, 1980, pp. 159-169.

Reddy, E.P., Smith, M.J., Canaani, E., Robbins, K.C., Tronick, S.R., Zain, S., and Aaronson, S.A.: Nucleotide sequence analysis of the transforming region and large terminal redundancies of Moloney-murine sarcoma virus. Proc. Natl. Acad. Sci., USA., 77: 5234-5238, 1980.

Rosen, S.W., Kaminska, J., Calvert, I.S., Ellmore, N., and Aaronson, S.A.: Ectopic production of "pregnancy-specific" beta-1 glycoprotein in vitro: discordance with three other placental proteins. Amer. J. Obstet. Gynecol., 137: 525-529, 1980.

Aaronson, S.A., Barbacid, M., Dunn, D.Y., and Reddy, E.P.: Genetic approaches toward elucidating the mechanisms of type-C virus induced leukemia. In Neth, R. (Ed.): Modern Trends in Human Leukemia, Vol. IV. Berlin/Heidelberg, Sringer-Verlag, 1981, pp. 455-459.

Nagao, K., Yokoro, K., and Aaronson, S.A.: Continuous lines of basophil/mast cells derived from normal mouse bone marrow. Science, 212: 333-335, 1981.

Cremer, K., Reddy, E.P., and Aaronson, S.A.: Translational products of Moloney murine sarcoma virus (MSV) DNA: Identification of proteins encoded by the MSV src gene. Journal of Virology. (In press.)

Merregaert, J., Barbacid, M., and Aaronson, S.A.: Recombinants between ts mutants of Rauscher murine leukemia virus and BALB:virus-2: genetic mapping of the Rauscher MuLV genome. J. Virol. (In press.)

Oroszlan, S., Barbacid, M., Copeland, T.D., and Aaronson, S.A.: Chemical and immunological characterization of the major structural protein (p28) of MMC-1, a rhesus monkey endogenous type C virus. J. Virology. (In press.)

Robbins, K.C., Devare, S.G., and Aaronson, S.A.: [illegible] genome organization of the integrated form of simian [illegible] virus. Proc. Natl. Acad. Sci. (In press.)

Srinivasan, A., Reddy, E.P., and Aaronson, S.A.: Abelson murine leukemia virus: molecular cloning of infectious integrated proviral DNA. Proc. Natl. Acad. Sci. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04941-09 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Steven R. Tronick	Microbiologist	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	P.R. Andersen	Senior Staff Fellow	LCMB	NCI
	K. Prakash	Visiting Fellow	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI
	E. Canaani	Expert	LCMB	NCI
	A. Varesio	Visiting Associate	LCMB	NCI
	K. Robbins	Expert	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to biochemically characterize retroviruses in order to understand the mechanisms by which these viruses induce cancer in their natural hosts. The role of these viruses in the etiology of human cancers is also under study. Studies currently in progress are the following: 1) biochemical characterization of replication-defective mammalian transforming viruses; 2) biochemical characterization of new isolates of retroviruses; 3) search for the presence of retroviral genes and gene products in human tumors.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. To biochemically characterize mammalian transforming retroviruses.
2. To biochemically characterize new isolates of retroviruses.
3. To determine the mechanisms of oncogenesis by retroviruses in their natural hosts.
4. To determine whether or not retroviruses play a role in the etiology of cancers of higher primates, including humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; molecular hybridization techniques using single-strand specific nuclease and hydroxyapatite chromatography to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing, and electron microscopy.

Major Findings:

Cloned DNAs representative of the onc genes of transforming retroviruses [Moloney murine sarcoma virus (MSV), BALB-MSV, Abelson leukemia virus (AbLV), woolly monkey sarcoma virus (WSV), and avian myeloblastosis virus (AMV)] were labelled to high specific activity with ^{32}P-dCTP and used to search for homologous sequences within human DNA. These studies were carried out collaboratively with K. Prakash, E.P. Reddy, P. Andersen, A Srinivasan, and K. Robbins. To date, a 2.5 kbp Eco RI fragment of human DNA homologous to Moloney-MSV onc was enriched by sequential RPC-5 chromatography and agarose gel electrophoresis and cloned in the phage vector Charon 16A. Libraries of cloned human DNA obtained from T. Maniatis yielded recombinant phage containing human DNA segments homologous to BALB-MSV (17 kbp); AbLV (4 kbp and 15 kbp); WSV (18, 17, and 7 kbp); and AMV (7 kbp and 2-3 kbp). Studies with each of these clones are now in progress and involve the following types of experiments: 1) Detailed restriction endonuclease mapping to determine whether viral onc-related sequences are present as a continuous or interrupted stretch of nucleotides. 2) Comparison of structures of human onc sequences to counterparts in respective retrovirus genome and normal cellular DNA of species of origin by using E.M. heteroduplex and hybridization with specific probes. 3) Preparation of hybridization probes of human onc-related and flanking sequences to study gene expression in human tumors. 4) Assessment of biological activity of cloned human onc-related sequences.

In collaboration with P. Andersen, the integrated form of BALB-MSV, a spontaneous transforming virus isolate from BALB/c mice, was cloned in a bacteriophage vector. The BALB-MSV genome was shown to contain direct LTRs, MLV-helper virus sequences, and a continuous segment of 0.6-0.9 kbp unrelated to MuLV. This segment was found to be derived from mouse cell DNA and is present in low copy number in the normal mouse cell genome. The helper-unrelated segment of BALB-MSV does not share homology with other mouse transforming retroviruses (Moloney-MSV and AbLV), but in studies carried out with R. Ellis and E. Scolnick, was shown to be related to the transforming gene of Harvey-MSV.

In collaboration with K. Robbins, subgenomic segments of the genomes of WSV, feline sarcoma virus, and Moloney murine leukemia virus have been subcloned in plasmid vectors. These segments are being used to study viral gene expression in normal and transformed cells of various mammalian species, and to determine which segments on the genome code for the various biological functions of these viruses.

Significance to Biomedical Research and the Program of the Institute:

Highly sensitive and specific biochemical probes for retroviral gene products generated in our studies have been extremely valuable in order to demonstrate the possible etiologic involvement of these viruses in human cancers and to study the role that retroviral gene products may play in normal cellular functions. The availability of molecularly cloned human gene fragments related to viral onc genes will greatly facilitate studies on the role the sequences play in the causation of human cancer.

Proposed Course:

A detailed biochemical and biological analysis of molecularly cloned human DNA analogues of retroviral onc genes is now under way.

Recombinant DNA techniques are being applied and further developed to study other mammalian transforming viruses and cellular genes involved in transformation.

Publications:

Canaani, E., Tronick, S., Robbins, K.C., Andersen, P. R., Dunn, C.Y., and Aaronson, S.A.: Cellular origin of the transforming gene of Moloney murine sarcoma virus. Cold Spring Harbor Symp. on Quant. Biol. 44: 727-734, 1980.

Reddy, E.P., Smith, M.J., Canaani, E., Robbins, K.C., Tronick, S.R., Zain, S., and Aaronson, S.A.: Nucleotide sequence analysis of the transforming region and large terminal redundancies of Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 77: 5234-5238, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04948-06 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Studies of Retroviral and Cellular Transforming Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Barbacid	Expert	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	K.C. Robbins	Expert	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	S. Pulciani	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

R. Huebner, LCMB, NCI
J. Rhim, LCMB, NCI
M.B. Gardner, USC
S. Rasheed, USC
P.K. Vogt, USC
C.J. Sherr, OAD, NCI
S. Ruscetti, LTVG, NCI
S. Orozlan, BCP, FCRC
R.V. Gilden, BCP, FCRC

LAB/BRANCH

Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are: 1) to search for retrovirus expression in cells and tumors of human origin; 2) to characterize the structural and functional properties of the transforming proteins of murine and feline sarcoma viruses; 3) to determine the mechanisms of sarcoma virus-induced cell transformation; 4) to identify cellular oncogenes of tumor cells by transmitting the transformed phenotype to normal cells with purified DNA; 5) to isolate these cellular oncogenes by molecular cloning techniques.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. To continue developing sensitive radioimmunoassays for the major structural proteins of mammalian retroviruses in order to (a) search for viral markers in human neoplasia and (b) characterize new retroviral isolates.

2. To identify the cellular substrates for the tyrosine-specific protein kinases associated with the transforming proteins of feline sarcoma viruses in an effort to understand the mechanisms by which these viruses cause malignant transformation.

3. To identify cellular oncogenes by transmitting the malignant phenotype of a variety of transformed cells, including those of human origin, by transfecting purified DNA into normal cells.

4. To isolate these oncogenic cellular genes by either plasmid rescue or related molecular cloning techniques.

Major Findings:

1. We have found that serologically distinguishable classes of B-tropic murine leukemia viruses (MuLV) have p30 proteins that differed from each other as well as from prototype N-tropic and xenotropic MuLVs. This polymorphism, along with the serological typing of the adjacent p12 polypeptide, suggests that a crossover within the region coding for p30 is involved in the generation of these B-tropic viruses.

2. We have found that normal feline embryo fibroblasts as well as feline cells of epithelial or lymphoid origin expressed low levels of a protein, NCP 92, antigenically related to the sarcoma virus-specific domain of the ST-FeSV transforming protein, ST P85. Normal cellular proteins cross-reactive with ST P85 were also detected in cell lines from various other mammalian species. These results suggest that the ST-FeSV sequences encoding for the sarcoma virus-specific domain of ST P85 originated from an evolutionarily conserved cellular gene expressed in cells of independent differentiation lineage.

3. We have found that the transforming protein of ST-FeSV, ST P85, possesses an associated protein kinase activity that specifically phosphorylates tyrosine residues. The physiological significance of this finding is illustrated by the fact that phosphotyrosine is an intrinsic component of ST P85. Furthermore, 5- to 10-fold higher levels of this unusual phosphorylated amino acid were present in ST-FeSV transformants than in uninfected control cells. Thus, phosphorylation of tyrosine residues appears to be associated with cellular transformation caused by Rous sarcoma virus and Abelson murine leukemia virus. These findings suggest that independent transforming virus isolates from birds, mice, and cats may utilize common pathways in exerting their oncogenic potential.

4. The major gene product of the Gardner-Arnstein (GA) strain of feline sarcoma virus (FeSV) has been characterized. This protein, designated GA P95, is a polyprotein that contains the amino-terminal moiety of the FeLV gag gene-coded precursor protein fused to a sarcoma virus-specific polypeptide. The latter appears to be highly related to the corresponding region of ST P85. Antibodies directed against GA P95 recognized the same feline cellular protein NCP 92 that cross-reacts with ST P85. In addition, GA P95 also has an associated protein kinase activity specific for tyrosine residues whose properties closely resembled those observed in ST P85.

5. We have studied the humoral immunity against the GA-FeSV gene product in natural FeLV-exposed and experimentally FeSV-inoculated house cats. We found that GA P95 was immunoprecipitated by GA-FeSV immune cat sera but not by any of the natural FOCMA-immune cat sera tested, after extensive absorption of anti-FeLV antibodies. These results suggest that FOCMA, as identified by immunofluorescence on FL74 lymphoid cells, is not identical to the FeSV transforming proteins.

6. DNA transfection techniques have been utilized to determine the regions of the ST-FeSV genome involved in malignant transformation. We have found that the 3.7-kbp 5'-end fragment of the ST-FeSV provirus is sufficient to transform NIH/3T3 fibroblasts. Enzymes that cleave the ST-FeSV provirus DNA within the feline leukemia virus gag gene sequences or within the fes gene abolished the transforming activity. Preservation of the proviral large terminal repeats was also required for transformation. Transformed NIH/3T3 cells obtained by transfection of total or subgenomic ST-FeSV DNA expressed normal levels of the ST-FeSV gene product ST P85 and of its associated protein kinase activity. Furthermore, these cells contained high levels of phosphotyrosine residues, a biochemical marker associated with cellular transformation induced by certain retroviruses including ST-FeSV. These results, taken together, strongly support the concept that only those ST-FeSV proviral sequences necessary for ST P85 expression are involved in malignant transformation.

7. We showed that the transformation-specific proteins of ST- and GA-strains of FeSV were immunologically related to those of the Fujinami and PRC II strains of avian sarcoma viruses. The antigenic determinants shared by these viral proteins have been mapped within their respective sarcoma virus-specific region, suggesting that the cellular insertions present in these avian and feline sarcoma viruses are related. These observations indicate that potentially oncogenic sequences have been conserved during the evolution of feline and avian genomes and have been independently acquired by two sets of sarcoma viruses.

Proposed Course:

1. To continue developing highly sensitive radioimmunoassays for retroviral proteins to search for viral markers in human tumors.

2. To identify the cellular targets for the protein kinase activities associated with FeSV gene products in order to understand the mechanisms of FeSV-induced oncogenesis.

3. To utilize genetic engineering techniques to isolate cellular genes with oncogenic properties.

Publications:

Barbacid, M., Beemon K., and Devare, S.G.: Origin and functional properties of the major gene product of the Snyder-Theilen strain of feline sarcoma virus. Proc. Natl. Acad. Sci. USA 77: 5158-5163, 1980.

Barbacid, M., Krakower, J., Bolognesi, D.P., and Aaronson, S.A.: The search for evidence of humoral immunity to oncoviruses in man. In Todaro, G.J., and Essex, M. (Eds.): Viruses in Naturally Occurring Cancers. New York, Cold Spring Harbor Laboratory Press, 1980, pp. 869-883.

Barbacid, M., Lauver, A.V., Long, L.K., and Devare S.G.: The major gene products of the Snyder-Theilen and Gardner-Arnstein strains of feline sarcoma viruses have a common cellular origin and similar functional properties. In Hardy, W.D., Essex, M., and McClelland, A.J. (Eds.): Feline Leukemia Virus. New York, Elsevier/North Holland Press, 1980, pp. 309-320.

Benade, L., and Barbacid, M.: Polymorphism among the major core proteins of C57BL B-tropic murine leukemia viruses. Virology 106: 387-390, 1980.

Gardner, M.B., Barbacid, M., Rasheed, S., Grant, C., and Aaronson, S.A.: Humoral immunity in natural FeLV-exposed and experimental FeSV-inoculated house cats. In Hardy, W.D., Essex, M., and McClelland, A.J. (Eds.): Feline Leukemia Virus. New York, Elsevier/North Holland Press, 1980, pp. 159-169.

Aaronson, S.A., Barbacid, M., Dunn, C.Y., and Reddy, P.E.: Genetic approaches toward elucidating the mechanisms of type-C virus induced leukemia. In Neth, R. (Ed.): Modern Trends in Human Leukemia, Vol. IV. Berlin/Heidelberg, Springer-Verlag, 1981, pp. 455-459.

Barbacid, M.: Cellular transformation by subgenomic feline sarcoma virus DNA. J. Virol. 37: 518-523, 1981.

Barbacid, M., Breitman, M.L., Lauver, A.V., Long, L.K. and Vogt, P.K.: The transformation-specific proteins of avian (Fujinami and PRC II) and feline (Snyder-Theilen and Gardner-Arnstein) sarcoma viruses are immunologically related. Virology 110: 411-419, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04950-06 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on Replication-Defective Mammalian Transforming Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.G. Devare	Visiting Associate	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI
	E.P. Reddy	Visiting Scientist	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI
	P.R. Andersen	Senior Staff Fellow	LCMB	NCI
	K.C. Robbins	Expert	LCMB	NCI
	A. Habara	Visiting Fellow	LCMB	NCI
	K. Prakash	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

K. Beemon, Salk Institute, San Diego, CA

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The molecular organization of simian sarcoma virus (SSV) cloned DNA by R-loop analysis revealed that this virus was generated by genetic recombination of helper simian sarcoma associated virus and about 1 kilobase pairs of cellular sequences. Construction of deletion mutants of SSV showed excellent correlation between physical localization of acquired sequences and its functional role in cellular transformation. 2. Rauscher murine leukemia virus and Moloney murine leukemia virus were shown to possess similar genomic complexity and thus their specificity in inducing B or T cell tumorigenesis may be a result of yet unknown mechanism. 3. Molecularly cloned BALB-murine sarcoma virus and Harvey-sarcoma virus DNA were shown to possess a homologous region of 0.75 kilobase pairs in their acquired transformation-specific sequences which play an important role in tumorigenesis. These results show that these two independent sarcoma virus isolates have been generated by acquisition of similar subsets of cellular genes as a result of genetic recombination and their transforming function may involve a similar molecular mechanism.

Project Description

Objectives:

To study genomic organization of replication defective mammalian retroviruses in order to define and localize gene(s) involved in malignant transformation of normal cells. Compare nucleic acid sequences with transforming potential from various retroviruses for identification of their origin and correlation between acquired cellular sequences. Analyze retrovirus-specific oncogenic sequences and their homologues in uninfected cells from diverse mammalian species.

Methods Employed:

"R-loop" analysis between molecularly cloned viral DNAs and viral RNAs to map cellular sequences acquired by helper retroviruses during generation of replication defective transforming viruses. Heteroduplex analysis between cloned viral DNAs to compare and correlate various independent retrovirus isolates and their cellular homologues. Molecular cloning and subcloning of retrovirus-specific genes to localize transforming sequences.

Major Findings:

1. Genomic organization simian sarcoma virus. Studies on several replication defective transforming retroviruses of avian and mammalian species have indicated that these viruses have been generated by genetic recombination between infectious helper viruses and specific cellular gene(s). The acquired cellular nucleotide sequences have been shown to bestow transforming potential to these recombinant retroviruses. Simian sarcoma virus (SSV), the only known retrovirus of primate origin with oncogenic capacity, was isolated from a tumor of woolly monkey. In an attempt to understand organization and characterization of the SSV genome, proviral integrated DNA from transformed nonproducer cells was molecularly cloned in Charon 16A strain of bacteriophage lambda. For localization of the cellular sequences acquired by helper simian sarcoma associated virus (SSAV) during generation of SSV, R-loop analysis between molecularly cloned SSV DNA and SSAV RNA was performed. The results of such analysis showed that during its generation, SSV acquired about 1 kilobase pairs (kbp) nucleotide sequences from cellular genome which lacked homology with helper SSAV RNA. Further, in the homologous region between SSAV RNA and SSV cloned DNA, one small (0.2 kbp) and one large (1.9 kbp) deletions of SSAV sequences in SSV genome were also observed. These results, along with the observation that SSV synthesizes gag gene proteins of 12,000 and 30,000 molecular weight, lead to localization of the acquired cellular sequences in SSV genome at 3.55 kbp from 5' end of genome, and further confirmed the evolution of SSV by genetic recombination of SSAV with cellular DNA sequences.

2. Transformation-specific sequences of SSV. For functional characterization of acquired cellular sequences in the SSV genome and their role in cellular transformation, two sets of deletion mutants of SSV cloned in pBR322 plasmid were constructed. On the basis of R-loop studies and restriction analysis the

acquired 1 kbp of cellular sequences are localized at 3.55 kbp from 5' end of the genome. In the first set of deletion mutants, sequences 1.6 kbp from 5' end to 3.15 kbp, which mainly contain part of gag gene and deleted pol gene (based on the analogy with other known retroviruses and the translational products of SSV genome), were deleted. In the second set, deletion was extended from 5' end 1.6 kbp to 3.85 kbp which deleted part of SSV genome, as described in first set of mutants, and in addition, about 325 bases of acquired cellular sequences (on the basis of R-loop and restriction analysis data). The transfection of these plasmid DNAs into NIH/3T3 cells revealed that even after deletion of part of gag and pol genes in SSV genome, the transforming capacity of first set deletion mutants was retained although at somewhat lower efficiency. On the contrary, deletion of part of acquired cellular DNA sequences completely abolished transforming potential in second set of mutants. These results corroborated the localization of transformation-specific sequences of SSV mapped by R-loop analysis. Further, these results suggested that acquired cellular sequences, including the first 325 bases, are essential for transforming capacity of SSV. Secondly, sequences in the central part of the genome may not be essential but may have some functional role in transformation by SSV.

3. Characterization of Rauscher murine leukemia virus (R-MuLV) molecularly cloned DNA. Previous studies have demonstrated that Moloney murine leukemia virus (Mo-MuLV) and R-MuLV induces tumors of T and B lymphoid cells, respectively. In order to investigate specificity of this tumor induction, R-MuLV integrated proviral DNA from normal rat kidney cells productively infected by the virus was molecularly cloned in Charon 4A strain of bacteriophage lambda. The R-loop analysis of the cloned DNA and R-MuLV RNA showed that the R-MuLV specific sequence of about 8.7 kbp is flanked on both sides with 2.9 kbp and 0.7 kbp cellular sequences, respectively. Heteroduplex analysis between cloned R-MuLV DNA and molecularly cloned Mo-MuLV DNA showed that R-MuLV DNA is closely related to Mo-MuLV DNA. This was further confirmed by heteropduplex formation between Moloney murine sarcoma virus (M-MSV) and R-MuLV which showed structural similarity to that reported for M-MuLV and M-MSV. The specificity of tumor induction by R-MuLV and Mo-MuLV thus may involve minor differences in the genomic complexity of these viruses which may not be visualized by heteroduplex analysis.

4. Molecular structure of Abelson murine leukemia virus. Ablelson murine leukemia virus (A-MuLV), induces lymphoid leukemia of B-cells in inoculated mice and transforms lymphoid, as well as fibroblast, cells in culture. Molecularly cloned A-MuLV DNA when heteroduplexed with Mo-MuLV cloned DNA showed homologous region of about 1.7 kbp and 0.7 kbp at 5' and 3' end, respectively, with non-homologous 3.0 kbp A-MuLV-specific sequences and 6.2 kbp Mo-MuLV DNA in between. These results confirm the results of R-loop analysis which demonstrate that A-MuLV has been generated by acquisition of murine cellular sequences by Mo-MuLV helper virus. It was noted that while R-loop analysis showed homologous sequences of about 1.3 kbp at 5' end with Mo-MuLV, our data indicates homology of 1.7 kbp in the same region. These observations are consistent with the fact that in proviral DNA molecules, additional sequences of long terminal repeats at 5' end are observed in each instance of integrated retroviral genome.

5. Acquisition of similar subsets of cellular sequences by BALB murine sarcoma virus and Harvey sarcoma virus. BALB murine sarcoma virus (BALB-MSV), a virus isolated from hemangiosarcoma of BALB mouse and Harvey sarcoma virus (HaSV), which originated in Chester-Beatty rats as a result of genetic recombination between Mo-MuLV and nucleotide sequences endogenous to rat genome, are two independent rodent isolates with transforming properties. Heteroduplex analysis of BALB-MSV cloned 6.2 kbp DNA and a 2.3 kbp DNA fragment containing transformation-specific cellular sequences from HaSV showed a 0.75 kb region of homology 4.35 kbp from 5' end of BALB-MSV cloned DNA. This homologous region has been shown to correspond with the acquired rat sequences in HaSV which confer transforming potential to the virus. The existence of 0.75 kbp region of homology between BALB-MSV and HaSV suggests that these two independent isolates acquired similar subsets of cellular sequences by the process of genetic recombination. Further, it also indicates that cellular transformation by these two isolates may involve a similar molecular mechanism.

Significance to Biomedical Research and the Program of the Institute:

1. Simian sarcoma virus (SSV) is the only known primate retrovirus with transforming potential. The results of R-loop heteroduplex analysis of the molecularly cloned SSV DNA and helper SSAV RNA has been useful in understanding the organization and characterization of the viral genome. The heteroduplex analysis data and the results of transfection by the SSV deletion mutants demonstrate that SSV was generated by acquisition of 1 kbp of cellular information and that this information is essential for cellular transformation. These results are of importance to our present understanding of the structure of a primate transforming retrovirus genome and the role of the acquired nucleotide sequences in cellular transformation.

2. The results of heteroduplex analysis between molecularly cloned R-MuLV and Mo-MuLV DNAs indicate that the specificity of B or T lymphoid cell tumor induction by these viruses may involve minor differences in genomic structure of these viruses. Alternatively, there may exist a yet unknown molecular mechanism by which specific lymphoproliferative diseases are induced by these closely related viruses.

3. Molecularly cloned BALB-MSV and HaSV DNAs showed a homologous region of 0.75 kbp in the acquired cellular sequences which have function in cellular transformation. These results favor the concept that these two independent sarcoma virus isolates of rodent origin have been generated by acquisition of similar subsets of cellular sequences. Further, these results have implications on the origin of these viruses and that their transforming function may involve a similar molecular mechanism.

Proposed Course:

Studies will be continued to determine genomic structure and oganization of mammalian retroviruses and their acquired cellular sequences. Experiments will be designed for understanding the molecular mechanism of tumorigenesis by these acquired nucleotide sequences and their cellular homologues in normal as well as malignant cells.

Publications:

Barbacid, M., Beemon, K., and Devare, S.G.: Origin and functional properties of the major gene product of the Snyder-Theilen strain of feline sarcoma virus. Proc. Natl. Acad. Sci. USA 77: 5158-5162, 1980.

Barbacid, M., Lauver, A., Long, L., and Devare, S.G.: The major gene products of the Snyder-Theilen and Gardner-Arnstein strain of feline sarcoma viruses have a common cellular origin and similar functional properties. In Hardy, W.D., Essex, M., and McClelland, A.J. (Eds.): Feline Leukemia Virus. New York, Elsevier/North Holland Inc., 1980, pp. 309-320.

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Devare, S.G.: Translational products of type D primate retroviruses. In Yohn, D.S., Lapin, B.A., and Blakeslee, J.R. (Eds.): Advances in Comparative Leukemia Research, 1979. New York, Elsevier/North Holland Inc., 1980, pp. 301-302.

Robbins, K.C., Devare, S.G., and Aaronson, S.A.: Molecular cloning and genome organization of the integrated form of simian sarcoma virus. Proc. Natl. Acad. Sci. USA, 1981 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04951-05 LCMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunological Characterization of Retrovirus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Dahlberg	Microbiologist	NCI	LCMB

COOPERATING UNITS (if any)

None

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

BALB/c mice have been immunized with retroviruses and used to produce hybridomas synthesizing monoclonal antibodies reactive to interspecies viral determinants. To date, all of the monoclonal antibodies produced which appear, in solid phase immunoassays, to react with two or more retroviruses, are IgM molecules which fail to immunoprecipitate any antigen. This suggests that antibodies having both interspecies reactivity and the necessary avidity for antigen are rare.

A biochemical and immunological characterization of a retrovirus recently isolated from goats has been initiated. This virus, termed caprine arthritis-encephalitis virus (CAEV), causes leukoencephalitis, interstitial pneumonia and chronic arthritis. We have shown that CAEV is morphologically and immunologically related to visna, a slow virus of sheep.

Project Description

Objectives:

1. To utilize hybridoma technology to develop monoclonal antibodies useful in characterizing retroviruses and differentiation antigens present on the surface membranes of transformed and normal cells.

2. To characterize, primarily by immunological means, a retrovirus recently isolated from goats which causes [illegible]encephalomyelitis and arthritis.

Methods Employed:

Light and electron microscopy, cell culture, virus growth and purification, column chromotography, electrophoresis, radioimmunoassays, and other preparative and analytical procedures.

Major Findings:

1. Previously described radioimmunoassays, which could detect interspecies immunological determinants common to retroviruses not closely related by other criteria, had limited usefulness due to either low sensitivity or a limited source of antiserum. It was felt that perhaps monoclonal antibodies could be produced which would have the necessary interspecies reactivities and eliminate some of the previously recognized limitations. Ideally, a small battery of immunoassays could then detect all, or nearly all, retroviruses and be useful in searching for new isolates.

BALB/c mice have been immunized, using a variety of schedules and routes, with several different retroviruses. The viruses principally used were SMRV, MPMV, RD114, and MLV, since they represent viruses distantly related to murine retroviruses, and, therefore, are likely to be most immunogenic. Following fusion of immune spleen cells with the P3 x 63 Ag8 myeloma cell line, hybrid cells were selected in HAT medium and screened with a solid phase immunoassay with 2 µg/well of the virus used for immunization. Hybrid cultures positive in this screen were expanded and assayed in a second solid phase immunoassay against 12-13 different retroviruses and an extract of the uninfected cell line used to grow the original virus.

In the past year, over 100 fusions were performed. The average number of clones obtained per mouse spleen was about 250, with a range of 0-700. Several hundred clones produced antibodies which were detected in the initial screen. Of these, about one-third had ceased antibody synthesis at the time of the second screen (about one week later), and most of the rest reacted against the control cell extract. Approximately 40 antibodies were found to react with more than one retrovirus extract and failed to react with uninfected cell extracts. These were further expanded, inoculated into pristane primed BALB/c mice and cryopreserved. Many of these hybridomas also stopped synthesizing antibody, and many reacted with control cell extracts when the higher titered ascites fluids were tested. Eleven hybridomas remain which, when tested in a solid phase assay, react with virus extracts but not cell extracts.

Since the original immunization had been done with concentrated virus preparations, which also contained varying levels of contaminating cellular and serum proteins, it was necessary to determine the specificity of these monoclonal antibodies by immunoprecipitation. None of these antibodies, which were all of the IgM class, were able to precipitate ^{35}S-methionine labeled extracts of virus-infected cells. The results of other laboratories have also indicated that many monoclonal antibodies, particularly IgM antibodies, precipitate the antigen that they recognize poorly or not at all. Thus, although these antibodies may well be virus specific, the inability to identify the viral (or other) antigen that they are directed against precludes their utilization in diagnostic assays. It was concluded from these results that the probability of obtaining monoclonal antibodies which not only had suitable wide specificity for different retrovirus antigens but also the avidity to be useful in conventional immunoassays was very low. It seems possible that, in terms of this technology, BALB/c mice may not find retroviruses very antigenic. Indeed, the reactivity of different strains of mice to a particular antigen may vary markedly, with BALB/c mice often being relatively poor responders.

2. It was recently reported that a disease syndrome in goats involving leukoencephalomyelitis, interstitial pneumonia, and chronic arthritis is caused by a retrovirus. This new isolate, termed CAEV (caprine arthritis-encephalitis virus) was shown to be immunologically related to visna virus, a retrovirus which can produce neurological disease and progressive pneumonia in sheep. Visna is considered a slow virus because following natural infection, clinically apparent disease requires months or years to develop. Visna virus (also called progressive pneumonia virus and maedi) is classified in the lenti virus group of retroviruses, as is equine infectious anemia virus (EIAV).

Visna and CAEV are economically significant because of high morbidity and mortality in infected herds (both viruses are found world wide) and are useful model systems for the study of slow virus disease in man. The purpose for studying CAEV has been two-fold: (1) to immunologically characterize the virus to determine its relationship to visna and other exogenous retroviruses of domesticated ungulates (including bovine leukemia virus) and develop sensitive immunoassays for diagnostic use, and (2) attempt to understand the mechanism by which CAEV and visna requires such a long time to cause pathological damage when the replication and cytopathogenicity *in vitro* are relatively efficient.

During the past few months, after a seed stock of CAEV was obtained, an ultrastructural comparison of CAEV to visna has been conducted and a biochemical and immunological comparison of CAEV and visna has been initiated. When visna and CAEV-infected cells are examined by electron microscopy, it is apparent that the two viruses are very similar, while distinct from type C and D retroviral morphology. Both viruses are variable in size, with budding virions ranging in diameter between 100 and 160 nm and with frequent aberrant structures, such as stalks and long arrays of membrane associated with viral material. The nucleoid in the budding virions, in both cases, is in close contact with the plasma membrane, and viral structures are also seen budding from ER membranes. The level of virus seen in CAEV-infected goat synovial cultures, however, is much lower than in visna-infected ovine fetal cornea cells. In the former case, although multinucleated cells have many budding virions, few extracellular virions are seen.

In an effort to obtain large amounts of virus for biochemical studies, a more permissive cell line was sought. Both Himalayan tahr ovary cells (a permanent cell line) and ovine fetal cornea cells were more permissive for CAEV, allowing the purification of moderate amounts of virus. CAEV has a density in sucrose of 1.15 g/ml, and its reverse transcriptase most efficiently utilizes Mg^{++} as divalent cation (similar to visna, EIAV, BoLV, MTV and type D retroviruses, but unlike type C retroviruses). The proteins of CAEV are similar to but not identical to those of visna with the major internal proteins being 28000 daltons versus 27000 daltons, respectively. Similarly, the smaller gag proteins differ considerably in size, while the glycoprotein of each virus is about 135000 daltons. Other virus-specific proteins have been difficult to identify due to the presence of contaminating cellular proteins in the virus preparations.

Effort is currently concentrated on the purification of CAEV gp135 and p28 to enable the development of radiommunoassays to sensitively measure the levels of these proteins for diagnostic purposes and to test for genetic relatedness between CAEV and other retroviruses. Preliminary experiments indicate that conventional procedures will be satisfactory, except that as for visna, conventional disruption procedures are not as efficient as for type C viruses.

Other experiments have been carried out, using both visna and CAEV infected cells, which detect virus-related proteins in virus and cell exracts. Both naturally occurring antibody from infected sheep and goats, and sera from rabbits immunized with CAEV, have been used to immunoprecipitate labeled extracts. The naturally occurring antibodies to both viruses predominantly recognize the viral glycoprotein, while the rabbit sera efficiently precipitate CAEV p28, p18 and p14, as well as a putative gag pol 180,000 dalton precursor and a 60000 dalton protein present in cell extracts but not viral extracts. Of particular interest are the extraordinarily high levels of both the p180 and p60 proteins in cell extracts. Type C virus-infected cells typically contain very low levels of pre-180 gag pol and somewhat higher levels of pre-75 gag and pre-65 gag. Pulse chase experiments with type C virus-infected cells indicate a rapid processing of precursor polyproteins to mature gag gene products and polymerase. For visna and CAEV-infected cells (even in these relatively permissive cells and in vitro growth conditions) such rapid processing does not seem to occur. Not only are the levels of putative precursors very high, but pulse chase experiments indicate that significant levels of label remain p180 and p60 for as long as 18 hrs following a 30' labeling period. These preliminary results suggest that processing of CAEV and visna polyproteins is unusually inefficient, even under fairly permissive conditions. These results may be of importance in helping to understand the nature of pathogenicity of these viruses. Various hypotheses have been advanced to explain how visna might take so long to lead to clinically apparent disease. Under natural conditions, CAEV infection also is characterized by long dormant periods and a high percentage of asymptomatic animals. Since antiviral antibody cannot be detected in visna lesions, and virus is not observed by EM, it has been argued that in most infected cells, the provirus is nonfunctional. Presumably, then, only the occasional activated cell will release virus, with a very slow, primarily cellular, immunological response occurring. Our results suggest that an additional mechanism may help to regulate the rate of virus production in vivo. Inefficient processing of CAEV and visna polyprotein would limit the release of virus even in turned on cells and would tend to slow the movement of virus within the animal.

Significance to Biomedical Research and the Program of the Institute:

1. The value of a battery of monoclonal antibodies capable of recognizing immunological determinants common to many diverse retroviruses would be significant. This potential benefit was considered great enough that a substantial effort was devoted to a search for antibodies considered likely to be rare. Our failure to obtain useful antibodies having both the desired specificity and ability to be used in immunoassays verifies the rarity, at least in the BALB/c repertoire, of lymphocyte reactive with retroviral interspecies determinants. The experience gained in hybridoma technology will be continued to be utilized, particularly in areas of tumor biology and cell surface markers.

2. Lenti viruses are exogenous retroviruses that apparently have evolved so rapidly that it is no longer possible to identify the species of origin. CAEV is a recently identified isolate that causes significant morbidity and mortality in goats. We have shown that CAEV is morphologically similar to visna virus, and is immunologically related to it. Our results suggest that the slowness of onset of the neurological and arthritic symptoms of in vivo infection may be partly due to inefficient processing of viral polyproteins. Such a mechanism might confer a selective advantage to a retrovirus which is not endogenous to its host species.

Proposed Course:

1. In the following year, an effort will be made to isolate hybridomas producing monoclonal antibodies identifying tumor-specific antigens and cell surface antigens specific to a particular cell type (differentiation markers). The specificity of these antibodies will be evaluated by fluorescent and immune electron microscopy, and tumor-specific antibodies will be tested for reactivity against normal cells to test the hypothesis that antigens which appear to be tumor specific may be differentiation antigens.

2. A variety of approaches will be undertaken to test further the hypothesis that inefficient cellular processing of CAEV (and visna) viral polyproteins may play a role in the slowness with which the virus replicates and leads to pathological damage in vivo. Immunoassays for gp135 and p28 will be developed and used to measure the level of viral protein being expressed in lesions present in infected animals and compared to expression in fresh explants and more permissive cell lines. The kinetics of processing in vitro will be investigated in greater detail and compared more directly to type C virus-infected cells. Efforts will also be made to alter processing in CAEV-infected cell extracts.

Publications:

Ablashi, D., Rice, J.M., Armstrong, G.R., Donovan, P.J., Sundar, K.S., Faggioni, A., and Dahlberg, J.: Enhancing effect of N-methyl-N-nitrosoguanidine on herpesvirus saimiri and Epstein-Barr virus. Proceedings International Workshop on Herpesvirus, 1981. (In press).

Dahlberg, J.E., Gaskin, J.M. and Perk, K.: Morphological and immunological comparison of caprine arthritis encephalitis and ovine progressive pneumonia virus. J. Virol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04967-06 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Genetic and Physiological Factors in Ontogeny and Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. T. Allen	Microbiologist	LCMB	NCI
Other:	A. K. Fowler	Head, Experimental Ontogeny Section	LCMB	NCI

COOPERATING UNITS (if any)

Research Inst. Infectious Disease U. S. Army, Ft. Detrick Frederick, Maryland 21701	Litton Bionetics Frederick Cancer Research Center Frederick, Maryland 21701

LAB/BRANCH

Carcinogenesis Intramural Program, Laboratory of Cellular & Molecular Biology

SECTION

Experimental Ontogeny Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The characteristics of bioregulatory substances associated with embryogenesis and fetal development are under investigation. Placental and fetal tissues, taken from mice in the period from the 12th day of gestation through late term, were homogenized under acid and neutral conditions. Acid extracts of both types of tissue contained growth factors which elicited a mitogenic response in quiescent cells of mouse and rat origin when tested in a semi-micro assay procedure developed during this reporting period. The growth factor(s) was distinguishable from epidermal growth factor (EGF) in two ways: it was active in cell strains which were refractory to EGF; and it failed to compete with ^{125}I-EGF for binding sites on A-431 human epidermoid carcinoma cells. Neutral extracts were similar in growth factor activity to acid extracts, but contained interferon-like antiviral activity as well.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

To develop and study model systems for evaluating the role of physiological and genetic factors, as they relate to carcinogenesis, through investigation of the growth regulatory factors associated with prenatal ontogeny and neoplastic development.

To establish in vitro bioassay systems to examine growth regulator interactions in normal and pathological growth control.

Methods Employed:

The induction of cellular proliferation by tissue-associated growth factors was examined by analyzing tissue-extracts for mitogenic activity. Extracts were prepared by homogenization in 4 ml of the indicated buffer per gram of tissue, followed by low speed centrifugation. A semi-micro mitogenic assay was developed in which indicator cells were propagated in microplates. Serial dilutions of tissue homogenate were tested by addition of 10 μl volumes to microplate cultures and scored for incorporation of ^{3}H-thymidine into an acid-insoluble form.

Epidermal growth factor (EGF) binding competition assays were done using human epidermoid carcinoma cells A-431. Purified EGF was obtained from a commercial source in both the iodinated and unlabeled forms. A-431 cells were pretreated with tissue homogenates or control materials before adding ^{125}I-EGF.

Mouse interferons were assayed on L_{929} indicator cells, challenged with vesicular stomatitis virus or the MM strain of encephalomyocarditis virus. Anti-viral activity was measured by four principal methods: inhibition of viral cytopathogenic effects; inhibition of virus-specific RNA synthesis; inhibition of virus yield; and inhibition of virus plaque formation.

Major Findings:

Growth factors were extracted from placental and fetal tissues in 50 mM acetic acid. Acid soluble components were neutralized with NaOH or by lyophylization. Extracts prepared in this way contained limited interferon activity when tested on L_{929} murine fibroblasts, as compared to placental extracts prepared in PBS. When tested on a variety of fibroblastic and epidermal murine cells, these extracts exhibited a mitogenic effect. The degree of mitogenic response was dependent on the condition of the cultured cells at the time of growth factor addition and on the inherent properties of each cell line. Maximal stimulation of cell cultures occurred in cultures propagated to confluency in medium containing 5 to 10% of fetal calf serum (FCS) which were subsequently depleted of serum growth factors by additional 48 to 72 hr incubation in medium of low serum content (0.2% to 0.3% FCS).

A semi-micro mitogenic assay was developed in which indicator cells were propagated as discussed above in microplates. Serial dilutions of tissue extracts were tested for growth factor activity by addition of 10 µl volumes to microplate wells. Incorporation of ^{3}H-thymidine into an acid-insoluble form was measured during a 4 hr exposure to the radio-labeled DNA precursor beginning 20 hrs after addition of the extracts. The mouse cell lines examined can be ordered in terms of decreasing response to placental growth factor(s) as follows: 3T3, BALB/c epidermal cells clone 25, BALB/c epidermal cells clone 41, L_{929}, JLSV9. SA_6 rat cells were also responsive to placental growth factor.

Examination of placental extracts prepared in Dulbecco's PBS modified to contain NaCl at 0.64 M indicates that the placental growth factor is readily solubilized in high salt. These extracts are higher in total protein concentration and contain interferon-like antiviral activity. No significant increase or decrease in growth factor activity has been found. Preliminary gel filtration analysis indicates the principal mitogenic factor is in the 10,000 to 20,000 molecular weight range.

Binding competition studies with iodinated EGF have shown no evidence for the presence of EGF in either acid or neutral placental extracts, suggesting the mitogenic activity is not due to this particular growth factor. In further support of this conclusion it was shown that cell lines which do not respond to EGF treatment undergo strong mitogenic response when treated with placental growth factor preparations.

Significance to Biomedical Research and the Program of the Institute:

The effects of exogenous growth factors on the proliferation and differentiation of fetal tissues under experimental conditions has been recognized for a number of years. More recently, EGF has been detected in fetal tissues and receptors for EGF have been reported on placental membranes. Cultured sarcoma cells are reported to release growth factor(s) which reduces their requirement for serum in the medium. The precise role that any of these factors play under natural conditions in ontogeny or in oncogenesis remains poorly understood. The goal of this project is to investigate the occurrence and interaction of these bioregulatory molecules. To this end, growth factor(s) and a growth factor antagonist (interferon) have been detected in placental and fetal tissues. Progress toward characterizing these factors and their occurrence during gestation has been made. With continued study, the role of these factors in the control of cell proliferation and differentiation may finally become elucidated.

Proposed Course:

Studies to purify and characterize the placental growth factor are in progress. Characterization will include comparison to other factors such as sarcoma growth factor, the somatomedins, multiplication stimulating activity (MSA), nerve growth factor (NGF), and fibroblast growth factor (FGF). A study of the receptor for placental growth factor is anticipated to characterize their nature in comparison to growth factor receptors under study in other laboratories.

Publications:

Allen, P. T., Strickland, J. E., Fowler, A. K., and Waite, M. R. F.: Antigenic determinants shared by the DNA polymerases of reticuloendotheliosis virus and mammalian type C retroviruses. Virology 105: 273-277, 1980.

Strickland, J. E., Saviolakis, G. A., Weislow, O. S., Allen, P. T., Hellman, A., and Fowler, A. K.: Spontaneous adrenal tumors in the aged, ovariectomized NIH Swiss mouse without enhanced retrovirus expression. Cancer Res. 40: 3570-3575, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04976-04 LCMB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Carcinogenesis of Mammalian Cells in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Katherine K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
OTHER:	Floyd M. Price	Biologist	LCMB	NCI
	Gary M. Jones	Microbiologist	LCMB	NCI
	Raymond Gantt	Research Chemist	LCMB	NCI
	William G. Taylor	Research Biologist	LCMB	NCI

COOPERATING UNITS (if any)

R. Parshad, Howard University College of Medicine; R.E. Tarone, Biometry Branch, NCI; J.R. Frost, Johns Hopkins Hospital, Baltimore, MD.; C.W. Boone, Al Hada Hospital, Saudi Arabia

LAB/BRANCH

Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.5	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to understand mechanisms of spontaneous and induced neoplastic transformation in cultured mammalian cells with emphasis on the use of human cells, particularly those of epithelial origin. The primary approaches are to characterize the environmental conditions that control and promote transformation and to determine the effect of some of these environmental factors on chromosome aberrations, DNA damage and repair of that damage. Other efforts are aimed at identifying characteristics of cells in culture which specifically correlate with their neoplastic potential in vivo.

PHS-6040
(Rev. 2-81)

Project Description

This project consists of the following studies:

Study 1: Light-induced chromatid damage in human skin fibroblasts in culture in relation to their neoplastic potential.

Background:

We found previously that mouse cells after prolonged culture show a significant increase in susceptibility to chromatid damage produced by a single 20 hr exposure to low-intensity visible (405 nm) light; furthermore, this increased susceptibility is associated with their spontaneous malignant transformation in culture. The chromatid damage, including breaks and exchanges, results directly or indirectly from the intracellular generation of clastogenic amounts of hydrogen peroxide (H_2O_2) and the derivative-free hydroxyl radical (•OH). Increased susceptibility to chromatid damage could result from greater initial chromosomal DNA damage or from impaired capacity to repair the damage. Greater initial damage could result from loss or inactivation of defense enzymes such as catalase and glutathione peroxidase (GSH:peroxidase) which decompose H_2O_2 and prevent •OH formation. We showed that no consistent decrease in catalase activity characterized the malignant mouse cells as compared with their normal counterparts. We then obtained cytogenetic evidence for a defect in DNA repair capacity in the malignant mouse cells as compared with their normal counterparts. From our experimental findings through the years, the so-called spontaneous malignant transformation of mouse cells in culture appears to be induced by specifically identifiable environmental factors such as a chemical component(s) in serum, visible light and oxygen, all of which cause chromosomal DNA damage. Furthermore, the progression to malignancy appears to be associated with a reduced capacity to repair the DNA damage produced by these environmental agents.

In contrast to rodent cells, which almost invariably undergo malignant transformation in culture, normal human cells are not only difficult to transform by carcinogenic agents but rarely undergo spontaneous transformation in culture. In the present study, we examined the possibility that human cells, like mouse cells, might show an increased susceptibility to light-induced chromatid damage associated with their neoplastic potential.

Objectives:

1) To evaluate in human cells the relationship between susceptibility to light-induced chromatid damage and neoplastic potential and 2) to compare normal human cells and their neoplastic derivatives with respect to activities of GSH:peroxidase and sensitivity to DNA breakage by H_2O_2.

Methods:

Leighton tubes each containing a coverslip were inoculated with cells. After 24 hr incubation, the experimental cultures were exposed at 37°C for 20 hr to low-intensity light (cool-white fluorescent, 4.6 W/m^2 at the growth surface). Control cultures were handled identically but shielded from light of wavelength <500 nm. For chromosomal analyses, the experimental and control cells were processed *in situ* on the coverslips by techniques described. Analyses were made on randomized coded preparations.

The GSH:peroxidase assays were carried out by the method of Paglia and Valentine. DNA damage in cells exposed to H_2O_2 was determined by alkaline coelution through polyvinyl chloride filters of DNA from a mixture of neoplastic and normal cells by the method of Kohn.

Major Findings:

As noted in last year's report skin fibroblasts from individuals with xeroderma pigmentosum (XP) and ataxia telangiectasia and from the XP sib (possible heterozygote), all genetically predisposed to a high risk of cancer, show an increased susceptibility to light-induced chromatid breaks after culture *in vitro*. The level of susceptibility attained is significantly higher than observed in 13 lines of fibroblasts from normal skin of donors ranging in age from 3 days to 92 years and from fetal skin tested at various population doubling levels. Two lines of normal skin fibroblasts tranformed by chemical carcinogens to neoplastic cells also show a significant increase in susceptibility as compared with their normal controls. These data indicated for human cells, as for mouse cells, an association between enhanced susceptibility to light-induced chromatid damage and neoplastic potential; this association is further supported by the high susceptibility of cells derived from a human adenocarcinoma.

Activities of the H_2O_2 scavenging enzyme GSH:peroxidase were similar in the paired normal and neoplastic cell populations. Also, cells of the paired lines were equally sensitive to DNA breakage by H_2O_2. These results suggest that the enhanced susceptibility associated with neoplastic potential may result from an impaired capacity to repair DNA rather than a greater initial sensitivity of the neoplastic cells to the damaging agent.

Study 2. Neoplastic transformation of human cells in culture associated with deficient repair of light-induced chromosomal DNA damage.

Background:

Chromatid breaks must represent unrepaired DNA double-strand breaks. These could arise directly during late S-G_2, indirectly during DNA synthesis on a template damage during G_1 or from single-strand breaks converted to double-strand breaks by a single-strand nuclease. Single-strand breaks, in turn, may arise directly or from failure of ligation during DNA repair.

Objectives:

To relate the incidence of light-induced chromatid breaks to the DNA repair capacities of normal human cells and their neoplastic derivatives.

Methods:

Since the repair of light-induced lesions could be influenced by the stage of the cell cycle at the time of insult, cells were exposed to light during G_1, S, and late S-G_2 and examined at the subsequent post-treatment metaphase. To study cells under physiologic conditions, agents to induce synchrony were not used. Exposure of cells (grown on coverslips in Leighton tubes) during late S-G_2 or G_1 was achieved by illuminating for 5 hr or for 5 hr followed by 15 hr in the dark, respectively. Since the chromatid damage is scored in metaphase cells, those cells examined after 5 hr of light would presumably be in late S-G_2 at the time of illumination, while those exposed for 5 hr followed by 15 hr in the dark would be in G_1. To illuminate during S phase, cells were exposed for 5 hr followed by 3 hr in the dark.

Major Findings:

Two lines of normal human skin fibroblasts and five derivative lines transformed in culture to neoplastic cells by chemical carcinogens were compared with respect to chromatid breakage produced by exposure to low-intensity fluorescent light (cool-white, 4.6 W/m^2) during G_1, S, and late S-G_2 periods of the cell cycle. Five additional normal human cell lines and a line derived from a lung adenocarcinoma were also examined for chromatid damage following light exposure during G_1 and late S-G_2. Only the neoplastic cells develop significant increases in chromatid breaks when exposed during S or late S-G_2 as compared with shielded controls. The chromatid damage produced by late S-G_2 exposure can be completely prevented by addition of mannitol, a scavenger of free hydroxyl radicals. Addition of caffeine (known to interfere with DNA repair) to the culture medium during late S-G_2 light exposure has no effect on the frequency of chromatid breaks in the neoplastic cells but significantly increases chromatid breakage in the normal cells. Since chromatid breaks must represent unrepaired DNA double-strand breaks, the present results indicate that in the normal cells, the late S-G_2 light-induced DNA damage is repaired by a caffeine-sensitive repair mechanism that is absent or deficient in the neoplastic cells. Furthermore, this damage is caused by free hydroxyl radicals generated during light exposure.

Light exposure during G_1 produces slight but not significant increase in chromatid breaks in the neoplastic as compared with normal cells. However, addition of caffeine to the culture medium immediately following G_1 light exposure during the S period significantly increases chromatid damage in the neoplastic cells only. These results suggest: (1) that the G_1 light-induced DNA damage in normal cells is repaired by a mechanism insensitive to caffeine, presumably excision repair, and (2) that the neoplastic cells are deficient in excision repair, but can handle G_1 light-induced damage during S by a caffeine-sensitive repair mechanism, presumably postreplication repair. It thus appears that the neoplastic cells are deficient in repair of both G_1 and late S-G_2 light-induced DNA damage.

Significance to Biomedical Research and the Program of the Institute:

In recent years, findings from several research areas implicate DNA damage and deficient repair in the process of malignant transformation. Because of the ubiquity of visible light and its diverse effects on mammalian cells in culture, including mutations, DNA strand breaks, DNA protein crosslinks, chromosome damage, neoplastic transformation in mouse cells, and the production of toxic, as well as growth-stimulating, substances in culture medium, photosensitization may play an important role in mechanisms of carcinogenesis. Skin cancer, the most prevalent form of cancer in the United States today, has been shown to be associated with exposure to sunlight. Further, our findings of an association between neoplastic potential and deficient DNA repair in human cells suggests that loss of DNA repair capacity may be a requisite step in neoplastic conversion.

Proposed Course:

Studies are in progress to extend these observations to responses of human carcinoma cells to x-irradiation, ultraviolet irradiation and treatment with carcinogens in efforts to determine whether a deficiency in DNA repair is a general property of neoplastic cells or cells of high risk individuals and, thus, may be a requisite step in the progression to malignancy. Studies on the molecular basis of the cytogenetic observations are also in progress.

Study 3. Development of epithelial cell systems for carcinogenesis studies.

Objectives:

1. To develop techniques for culturing normal human epithelial cells for use in carcinogenesis studies and to develop a strategy for transforming human skin epithelial cells with physical and chemical environmental agents (see report by Dr. W. Taylor, Z01 CP 04978-04 LCMB).

2. To evaluate the role of H_2O_2 in inducing malignant transformation of mouse epidermal cells in culture and the role of added catalase in preventing karyotypic instability and spontaneous malignant transformation.

3. To examine and monitor the susceptibility of mouse epidermal cells to chromatid damage by x-irradiation, ultraviolet irradiation and visible light and to assess by autoradiography their capacity to repair UV- and carcinogen-induced DNA damage.

Accomplishments to date:

Mouse epidermal cells are being passaged in culture under various experimental conditions to meet these objectives.

Significance to Biomedical Research and the Program of the Institute:

Since most human cancers are carcinomas, it is important to develop neoplastic transformation systems with epithelial cells.

Proposed Course:

These projects will be continued to meet the objective of developing transformation systems with epithelial cells.

Study 4. Fundamental cytologic characteristics of carcinogenic change (collaboration with C.W. Boone and J.K. Frost).

Background:

Previous studies from this laboratory have identified and documented morphologic and cytologic criteria of neoplastic transformation of rodent cells in culture. These, in general, differ from those criteria used by practicing cytopathologists in the diagnosis of human cancer from Papanicolaou-stained preparations. Since exfoliative cytology, as applied in the Papanicolaou test, has proved to be a powerful tool in the early diagnosis of human cancer, and because it concerns largely nuclear morphology, we wished to evaluate the additional criteria used in this procedure.

Objectives:

1. To determine whether standard cytological procedures used by practicing cytopathologists can be used to determine different degrees of neoplastic potential of cultured cells growing attached to coverslips.

2. To determine which of these criteria are artifacts of fixation and which can be seen in living cells.

Methods:

Our paired normal and tumorigenic rodent and human cells have been grown on coverslips and fixed here, and then Papanicolaou stained in the laboratory of Dr. Frost at Hopkins Hospital. A kit of unknowns is being evaluated by a group of 8 outstanding cytopathologists in the country.

Accomplishments to date:

Five of the 8 cytopathologists have been contacted and 2 have completed their evaluations. A review of the first of these indicates almost completely correct diagnosis. Living preparations of the cells are being photographed here to meet the second objective.

Significance to Biomedical Resarch and the Program of the Institute:

With increasing emphasis in our program on human epithelial cells for carcinogenesis studies, it is important to establish cytologic and other criteria of malignant transformation in human epithelial cells and to attempt to analyze the underlying molecular basis for the morphologic changes.

Proposed Course:

Cytologic criteria found to be of diagnostic value will be applied to transformed human epithelial cells in culture as these become available.

Publications:

Parshad, R., Sanford, K.K., Jones, G.M., Tarone, R.E., Hoffman, H.A., and Grier, A.H.: Susceptibility to fluorescent light-induced chromatid breaks associated with DNA repair deficiency and malignant transformation in culture. Cancer Res. 40: 4415-4419, 1980.

Parshad, R., Taylor, W.G., Sanford, K.K., Camalier, R.F., Gantt, R., and Tarone, R.E.: Fluorescent light-induced chromosome damage in human IMR-90 fibroblasts: role of hydrogen peroxide and related free radicals. Mutation Res. 73: 115-124, 1980.

Sanford, K.K., Boone, C.W., Merwin, R.M, Jones, G.M., and Garrison, C.V.: The plate implant as a bioassay for the neoplastic potential of cultured cells. Int. J. Cancer 25: 509-516, 1980.

Camalier, R.F., Gantt, R., Price, F.M., Stephens, E.V., Baeck, A.E., Taylor, W.G., and Sanford, K.K.: Effect of visible light on benzo(α)pyrene binding to DNA of cultured human skin epithelial cells. Cancer Res. (in press)

Tucker, R.W., Meltzer, M.S., and Sanford, K.K.: Susceptibility to killing by BCG-activated macrophages associated with "spontaneous" neoplastic transformation in culture. Int. J. Cancer (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04977-04 LCMB

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Primary Biochemical Alterations Leading to Neoplastic Conversion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.R. Gantt	Research Chemist	LCMB	NCI
OTHER:	K.K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
	W.G. Taylor	Research Biologist	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular & Molecular Biology

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This program is designed to identify primary chemical and structural alterations in DNA leading to neoplastic transformation in human cells and rodent cells and quantitate the repair response of the cells to agents which induce the primary alterations. The immediate objectives are to develop methods to transform human epithelial cells and to compare the type of lesions caused by low level (nontoxic) insults to DNA with high level (toxic) insults and measure the cell repair response to these lesions. The lesions currently of most interest are DNA-protein crosslinks including both their induction and repair.

Project Description

Objectives:

To identify primary changes in cellular and viral nucleic acids during photochemical, chemical, and viral carcinogenesis and to develop and apply techniques for assaying the repair responses of the cells. The use of human epithelial cells is emphasized where it is technically feasible and the study of the induction, consequences, and repair of DNA-protein crosslinks is currently stressed.

Methods Employed:

The standard laboratory techniques for measuring single and double strand DNA breaks, repair synthesis, base damage, base adducts, and crosslinks are used with the usual adaptations and innovations.

Major Findings:

1. Repair of DNA-protein crosslinks. The reagent *trans*-Pt (*trans*-diaminodichloroplatinum) induces DNA-protein crosslinks. We are investigating the mechanism(s) by which mammalian cells repair these crosslinks. The conditions chosen for these studies minimize cell toxicity to increase the relevance of the data to conditions of chronic exposure. The results to date (manuscript in preparation) include: (1) In mouse L1210 cells the lesions induced by a 2 hour exposure to 20 mM *trans*-Pt are completely repaired in 24 hours. (2) The repair is not inhibited by caffeine which indicates the caffeine-sensitive postreplication repair system is not involved. (3) In the presence of cycloheximide (1 μg/ml) repair is inhibited. This suggests the requirement for protein synthesis and/or cell cycling since blocking protein synthesis also blocks DNA synthesis. (4) Repair of the crosslinks is inhibited in conditioned medium. Protein synthesis was shown to continue in conditioned medium at about 25% the rate of cells growing exponentially in fresh medium. The rate in conditioned medium was 5X the rate in fresh medium plus cycloheximide. (5) *Trans*-Pt, in contrast to *cis*-Pt, immediately (less than 1 hour) blocks progress of the DNA replication fork. *Cis*-Pt requires more than four hours. After the immediate blockage, there is a slow recovery in the presence of *trans*-Pt while in the presence of *cis*-Pt there is a continued decline and finally cell death undoubtedly due to formation of lethal DNA-DNA crosslinks. These results indicate that repair of DNA-protein crosslinks is cell cycle dependent in L1210 cells and suggest S phase may be required.

2. Interaction of trans-Pt and cis-Pt with DNA. The very rapid decline in DNA replication fork migration rate caused by *trans*-Pt compared to the slow decline in rate caused by *cis*-Pt suggests there may be a large difference in reactivity. To test this possibility, two approaches were used. First, lambda phage DNA was reacted with *cis*- and *trans*-Pt under various conditions and its mobility in acrylamide gels was determined to estimate crosslink formation.

Second, purified, ^{14}C-labeled mouse DNA was reacted with cis- and trans-Pt under various conditions and its binding to a nitrocellulose filter was determined to compare DNA-protein crosslink formation. The results from these experiments indicate the following: (1) Reaction of trans-Pt with lambda DNA in the presence of medium forms crosslinks much more rapidly than reaction with cis-Pt. The electrophoretic mobility assay does not distinguish between DNA-protein and DNA-DNA interstrand crosslinks. In the absence of medium there is a large reduction in crosslinking, but trans-Pt still crosslinks faster than cis-Pt. (2) Consistent with the electrophoretic results, purified mouse DNA is retained much more completely on a nitrocellulose filter after reaction with trans-Pt compared with cis-Pt. These results show that trans-Pt reacts much faster than cis-Pt to form DNA-protein crosslinks and suggests that trans-Pt in the absence of protein may form DNA-DNA interstrand crosslinks faster than cis-Pt.

Significance to Biomedical Research and the Program of the Institute:

Reports of others show that DNA-protein crosslinks (trans-Pt induced) increase sister chromatid exchanges and transform 10T-1/2 mouse cells, observations which indicate important perturbations of DNA. However, DNA-protein crosslinks are reported to be mutagenic. These observations taken together with our finding that repair of the crosslinks is cell-cycle dependent have two important aspects. First, it strengthens the idea that DNA-protein crosslinks (which are induced by a wide variety of carcinogens including x-rays, light, and many chemicals such as benzopyrene, methylmethane sulfonate, AAF, etc.) may play a role in epigenetic events leading to malignant transformation. Second, DNA-protein crosslinks may accumulate with time in noncycling cells of animals if repair is dependent on cycling and not subject to the cycle independent excision repair mechanism. In animals this accumulation would be expected to impair the function of organ systems containing significant numbers of nondividing cells, particularly at the level of induction of protein synthesis and production of messenger RNA; a general decline of organ response would ensue.

The toxicity and antitumorigenic activity of cis-Pt is thought to be due to its ability to form DNA-DNA crosslinks. The dramatic difference between cis-Pt and trans-Pt in these properties is generally attributed to a steric configuration which inhibits DNA-DNA interstrand crosslink formation by trans-Pt. The results reported here suggest an alternative explanation based on the apparent large difference in rates of reaction. Due to the "trans effect" in Pt compounds (chemical groups trans to each other mutually influence one another's reactivity, while groups cis to each other have little effect) the initial chloride in trans-Pt will react rapidly while the second chloride will tend to react at the rate of the chlorides in the cis-Pt. Therefore, the first reactive chloride of trans-Pt (or the aquated form) would be largely displaced by -SH and $-NH_3+$ groups before reaching the DNA of a cell. The second chloride (or aquated OH) would then react much more slowly than the first chloride allowing substantial exposure time and reaction with DNA. This would result in many adducts to DNA (including proteins) compared with DNA-DNA crosslinks. Since in cis-Pt both chlorides (or aquated OH's) react comparatively slowly, there is a much higher probability that unreacted cis-Pt will reach DNA in the nucleus before any reaction and thus allow both groups on one Pt to react with DNA to give interstrand crosslinks. This concept may be useful in optimizing the design of Pt compounds for chemotherapeutic purposes.

Proposed Course:

A. Establish whether human cells require cell cycling to repair DNA-protein crosslinks.

B. Determine the phase(s) of the cycle when DNA-protein crosslinks are repaired.

C. Establish clearly whether an inducible system is required for crosslink repair.

D. Simplify the assay procedure and develop alternate modes of crosslink production.

E. Establish the relative formation of DNA interstrand crosslinks by cis- and trans-Pt using purified DNA.

F. Look for the accumulation of DNA-protein crosslinks in organs of animals and compare cycling cells with noncycling cells.

Publications:

Chepelinsky, A. B., Gantt, R., and Wivel, N.: Presence of RNA methylases in intracisternal A particles purified from a mouse plasma cell tumor. European J. Biochem. 103: 339-347, 1980.

Parshad, R., Taylor, W. G., Sanford, K. K., Camalier, R. F., Gantt, R. and Tarone, R. E.: Fluorescent light-induced chromosome damage in human IMR-90 fibroblasts. Role of hydrogen peroxide and related free radicals. Mutat. Res. 73: 115-124, 1980.

Price, F. M., Camalier, R. F., Gantt, R., Taylor, W. G., Smith, G. H. and Sanford, K. K.: A new culture medium for human epithelial cells. In Vitro 16: 147-158, 1980.

Camalier, R. F., Gantt, R., Price, F. M., Stephens, E. V., Baeck, A. E., Taylor, W. G. and Sanford, K. K.: Effect of visible light on benzo(α)-pyrene binding to DNA of cultured human skin epithelial cells. Cancer Research 41: 1789-1793, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04978-04 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Growth, Nutrition, and Neoplastic Transformation of Mammalian Cells In Vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.G. Taylor	Research Biologist	LCMB	NCI
OTHER:	K.K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
	R.R. Gantt	Research Chemist	LCMB	NCI

COOPERATING UNITS (if any)

R. Parshad, Howard University College of Medicine

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
In Vitro Carcinogenesis

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term objective of this program is to understand the mechanism(s) of neoplastic transformation in cultured mammalian cells, particularly human cells. Studies described in this report address these intermediate goals: (1) modulation of proliferation in epithelial cell cultures as a function of calcium or dissolved oxygen concentrations; (2) induction of cellular injury by visible light or drugs, and subsequent repair; and (3) assessment of the undersurface morphology of nonneoplastic and neoplastic cells by interference-reflexion microscopy. Both calcium and oxygen are critical for sustained epithelial cell proliferation; calcium concentrations required by mass cultures ($\sim 5 \times 10^5 - 10^6$ cells) are higher than earlier reports with clonal systems. Though oxygen may cause cell injury, both human and monkey epithelial cells exhibit a critical oxygen requirement for proliferation which is much greater than that reported for fibroblasts. Examination of the undersurface of neoplastic and nonneoplastic cells is in progress.

Project Description

STUDY 1: Modification of epithelial cell profileration by calcium or oxygen concentration.

Objectives:

Most solid human tumors are carcinomas which arise in tissues rich in epithelium. Thus, the use of epithelial cells is preferred for experimental carcinogenesis studies. Two epithelial cell systems are in use in this laboratory: primary or low passage human keratinocytes which can be grown in medium NCTC 168 without a feeder layer, and line NCTC 8939, a derivative of LLC-MK_2 rhesus monkey kidney cells, which can be grown in serum-free medium. Our strategy for transformation of human epithelial cells includes temporary alteration of the cellular environment to maximize the impact of potential transforming agents. One objective of studies during this last year was to assess human epithelial cell proliferation and population density at different calcium concentrations. In separate studies, oxygen requirements for continued proliferation and adhesion were quantified with NCTC 8939.

Major Findings:

1. Ionic calcium concentration. Horse serum-supplemented medium NCTC 168 reproducibly yields confluent epithelial cell sheets from explants of neonatal foreskin. Striking morphologic differences are seen as a function of calcium concentration. Explants incubated in calcium-free medium NCTC 168 containing 10% v/v chelex-treated horse serum (residual calcium concentration ≈ 0.02 mM) form colonies of highly polygonal, refractive cells reminiscent of continuous or transformed lines. With time the colonies deteriorate with little net increase in colony diameter. With 0.15 mM Ca^{++}, cell morphology gradually becomes aberrant and outgrowth from explants retarded. At 0.3 - 0.5 mM Ca^{++}, the intercellular space appears slightly greater than at 2 mM, but the epithelial morphology is retained; examination of the colony periphery suggests a generally increasing degree of compactness--hence, population density--with increasing Ca^{++} concentration. Anucleated squame formation is apparent as early as 11 days in culture at 2 mM Ca^{++} and, perhaps, at 0.5 mM as well. Because of the difficulty in obtaining replicacy and quantifying cell numbers in explant cultures, we adapted procedures for preparation of suspensions of epithelial cells directly from foreskin. Suspensions are used to inoculate replicate cultures in collagen-coated flasks. Improved methods for measurement of cell growth by enumeration of trypsinized cells or isolated cell nuclei were developed. In an effort to increase the fraction of cells in DNA synthesis, and in view of our earlier findings and reports that low Ca^{++} enhances both plating efficiency and colony growth, we examined the influence of Ca^{++} concentration on cell yield in mass culture. The chelex-treated serum as compared with untreated serum yielded a comparable population density when tested at equivalent Ca^{++} levels (1 mM). In all experiments to date, a concentration of 0.15 mM fails to support continued growth, whereas concentrations of 0.30, 1, and 2 mM always support continued growth with 1 mM usually sufficient to high cell yield in both primary and secondary cultures.

2. Dissolved oxygen concentration (PO_2). Earlier studies in this laboratory with primary human foreskin keratinocytes and monkey kidney epithelial cells (LLC-MK_2) showed that proliferation rate and population density are strikingly sensitive to growth medium PO_2 (submitted for publication). Direct measurement showed that LLC-MK_2 cells require > 70 mm Hg for sustained proliferation and concomitantly utilize available oxygen rapidly until this threshold value is reached. Deliberately lowering the PO_2 to values typical of venous blood retards epithelial cell proliferation. More recent studies show the retarded proliferation rate at a lowered PO_2 is not the result of diffusion gradient formation (i.e, stratification of the oxygen supply), is reversible, and cannot be explained by an accelerated loss of cell viability. The adhesion of primary human epithelial cells, and to a lesser extent the LLC-MK_2 cells, is diminished at the lowered PO_2. Since cell adhesion is energy (thus oxygen) dependent and mitosis probably requires mechanical traction which is dependent upon firm adhesion, human fibronectin HFN was used to enhance cell-growth surface adhesion; however, a microcarpet of HFN failed to stimulate proliferation in cells retarded by an insufficient growth medium PO_2.

Significance to Biomedical Research and the Program of the Institute:

In vivo the homeostatic control exertion by the microvascular network, transcapillary diffusion gradients, and the intracellular cytochrome oxidase system maintain tissue oxygen tensions at relatively low levels and thereby minimize cell injury resulting from accumulation of deleterious oxygen intermediates, free radicals, and hydroperoxides. Yet our data show that proliferation of epithelial cells in culture requires a significantly higher dissolved oxygen concentration. This suggests that epithelial cells must have "defense mechanisms" which mitigate oxidative damage. This inference agrees with an earlier finding that epithelial cells are less sensitive to visible light-induced H_2O_2 production.

Ionic calcium has been proposed as a "switch" epithelial cell proliferation and differentiation. Studies to date in this laboratory show that the growth rate of epithelial cells in mass cultures deprived of adequate calcium is reduced and that best results are achieved with calcium concentrations significantly higher than reported in other laboratories.

Proposed Course:

Most pertinent studies on dissolved oxygen concentration and proliferation have been completed. As time permits, efforts will be made to determine if the retarded proliferation of NCTC 8939 at lowered PO_2 values is associated with G_1 restriction point control of cell division. Calcium, dissolved oxygen, and other essential nutrients will be varied in ongoing studies to transform human epithelial cells.

STUDY 2. Induction of cellular injury or death following exposure to visible light or cytotoxic drugs.

Objectives:

Visible light and oxygen are ubiquitous environmental agents which interact with cells in culture and their surrounding milieu. A 3-20 hr exposure of rodent cells to fluorescent light at an intensity comparable to that in the average laboratory results in chromatid damage. Exposure of cell-free culture medium to light results in hydrogen peroxide production, and the addition of catalase to cell cultures during light exposure reduces the frequency of chromatid breaks and exchanges.

These studies were designed to (a) determine whether chromosomes of normal human fibroblasts are sensitive or resistant to light-induced chromatid damage, (b) evaluate further the cytocidal influence of light-exposed NCTC 168 for low passage fibroblasts and determine if this was associated with hydrogen peroxide production, (c) test whether one primary event in cell killing was DNA strand breakage and, (d) evaluate repair of cell injury induced by visible light or drugs.

Major Findings:

1. Exposure of normal Phase II human fibroblasts to low-intensity fluorescent light causes chromatid breaks, which can be prevented by addition of catalase during light exposure. A similar frequency of chromatid breaks is seen when exogenous hydrogen peroxide is added in nonlethal concentrations. Light-induced damage can be decreased, but not prevented, by addition of sodium selenite (Ref. 1). It is noteworthy that glutathione peroxidase is a selenoenzyme which is induced by sodium selenite and functions in an oxidation-reduction system with NADP/NADPH to regulate intracellular hydrogen peroxide levels. See also Dr. Sanford's report No. Z01 CP 04976-04 LCMB - STUDY 1.

2. Fibroblasts illuminated in medium NCTC 168 are rapidly killed, whereas primary foreskin epithelial cells appear unaffected. The effective wavelength(s) responsible for this cytocidal effect is in a range between 365-405 nm, i.e., the visible range. Illumination of cell-free NCTC 168 generates stable, cytocidal photoproducts. Approximately 60% of the cell killing is reversed by catalase and H_2O_2 added at levels detected in the illumination growth medium (Ref. 2) may be cytocidal, depending on the exposure period. Organic peroxides and free radicals also will influence survival.

3. Collaborative studies with Dr. Gantt in which human fibroblasts are injured deliberately with H_2O_2 or drugs such as platinum coordination compounds are in progress. DNA strand breakage, DNA repair and cell survival are being monitored.

Significance to Biomedical Research and the Program of the Institute:

Solar energy peaks in the visible range (~ 470 nm), and the radiant energy in the 400-500 nm band is greater than in any subsequent 100 nm band. Skin filters ultraviolet light, but significant amounts of visible light penetrates the superficial layers. Evidence that visible light is mutagenic and promotes neoplastic transformation of otherwise untreated mouse cells is increasing (Cancer Res. 39: 929-933, 1979; Photochem. Photobiol. 31: 135-141, 1980; Science 207: 1209-1211, 1980). Although artificial light is less intense than solar energy, an ever increasing amount of fluorescent lighting is being used.

That fluorescent light produces chromatid damage in human cells indicates that light-induced genetic lesions are not limited to mouse cells but represent a general biologic response. Cell culture systems can be used to discriminate between the induction and repair of genetic lesions irrespective of the inducing agent. This strategy, within a controlled environment, will allow a better understanding of the mutagenic and potentially carginogenic events which precede neoplastic transformation.

Proposed Course:

1. The roles of catalase and glutathione peroxidase and other cellular defense mechanisms need further study.

2. An efficient means of minimizing light/drug-induced damage or enhancing repair of such damage also needs further consideration. Earlier studies with agents designed to protect against radiation damage (AET, BHT, etc.) did not appear promising. In view of the results of subsequent studies, this approach will be reviewed.

STUDY 3. Evaluation of cell undersurface adhesion sites as a marker for tumorigenic potential.

Objective:

Cell shape changes characterize responses to deleterious agents or growth conditions and frequently accompany the acquisition of neoplastic potential by cells in culture. Following cell retraction due to injury or the morphologic alterations associated with transformation, the total area of cell membrane which remains in apposition to the growth surface is reduced. An earlier study showed that neoplastic mouse fibroblasts have higher rates of locomotion and exhibit a more random pattern of movement when compared to nonneoplastic cells from the same clone (Sanford et al., ECR 109: 454, 1977). One possible explanation is that neoplastic fibroblastics have a reduced number of sites with which they adhere to the growth surface or, alternatively, that the sites of attachment on the undersurface of a neoplastic cell are less adhesive. Conceivably, this would contribute to the transformed cell's ability to metastasize and develop invasive neoplasms.

To evaluate this hypothesis, we will examine the undersurface of paired neoplastic and nonneoplastic cells.

Findings to Date:

Though the general principles for visualizing undersurface morphology have been published, a satisfactory photomicrography system has required an empirical approach which integrates cellular, microscopic, and photographic procedures.

Preliminary studies, in fact, suggest a difference in adhesion characteristics, but more data is required before valid conclusions can be drawn.

Significance to Biomedical Research and the Program of the Institute:

Recently it has become apparent that the apical (upper) and basal (in apposition with the culture flask) surfaces of cells in culture are functionally quite different. Moreover, the interrelationships between microfilament bundle attachment to the cell membrane, locomotion morphology, and virus infection are beginning to emerge, and all are related to the way in which cells attach to a growth surface of other cells and how cell growth is controlled. Interference-reflexion microscopy is a relatively new tool which can be used in conjunction with fluorescent microscopy to characterize cell adhesion sites of anchorage-dependent and-independent cells.

Proposed Course:

Initially, a randomized sampling of cells of known tumorigenic potential will be examined. The association of cell shape changes with adhesion sites and cytoskeletal components will also be examined. The image of the undersurface can be digitalized, and we anticipate that computerized image analysis can be used to quantify differences between normal and neoplastic cells.

Publications:

Taylor, W.G., Camalier, R.F., and Taylor, M.J.: A spectrophotometric assay for hydrogen peroxide in tissue culture medium. TCA Manual 5: 1081-1086, 1979.

Parshad, R., Taylor, W.G., Sanford, K.K., Camalier, R.F., Gantt, R., and Tarone, R.E.: Fluorescent light-induced chromosome damage in human IMR-90 fibroblasts. Mutation Res. 73: 115-124, 1980.

Camalier, R.F., Gantt, R., Price, F., Stevens, E.V., Baeck, A.E., Taylor, W.G., and Sanford, K.K.: Effect of visible light on benzo(α)pyrene binding to DNA of cultured human skin epithelial cells. Cancer Res. 41: 1789-1793, 1981.

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U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04994-04 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

MMTV Genes in Mouse Strains with a Low, Moderate or High Incidence of Mammary Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William Drohan	Sr. Staff Fellow	LCMB	NCI
Others:	Jeffrey Schlom	Section Chief	LCMB	NCI
	Robert Callahan	Microbiologist	LCMB	NCI
	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
	Steven Tronick	Microbiologist	LCMB	NCI
	Gilbert Smith	Microbiologist	LMB	NCI
	Michael Potter	Section Chief	LCBGY	NCI

COOPERATING UNITS (if any)

The Jackson Laboratory, Bar Harbor, Maine
Baylor College of Medicine, Houston, Texas

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We investigated the relationship between mammary cancer and the presence of specific endogenous and infectious mouse mammary tumor virus (MMTV) genetic elements in murine tissue. Using hybridization kinetics, restriction endonuclease analysis and the Southern blot technique we have studied the MMTV proviral content of tissues from mice with a low (C3H/StWi), moderate (BALB/cV), or high (GR) incidence of spontaneous mammary tumors. Our studies in C3H/StWi mice have shown that amplification of endogenous MMTV sequences or infection with an exogenous virus does not appear to be involved in tumorigenesis. Analysis of mammary tumor DNA from BALB/cV demonstrated integrated MMTV specific DNA not found in normal organs from these mice. We have subsequently isolated and characterized a new MMTV variant from these mice. This new virus differs biochemically and immunologically from other MMTV isolates. In the high tumor incidence GR strain, we have identified a single endogenous GR provirus which segregates with the incidence of mammary cancer in crosses of inbred mice, and appears to be responsible for inducing the high incidence of mammary cancer seen in this strain.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To identify and utilize inbred strains of *M. musculus* with low, moderate, and high incidences of spontaneous mammary adenocarcinomas to determine what role, if any, that germline and infectious variants of MMTV play in tumorigenesis. To do this we utilized restriction endonuclease analysis and the Southern blot technique to develop molecular markers that could be used not only to distinguish the many endogenous variants of MMTV from one another, but also from exogenous variants of MMTV which are maintained as infectious viruses in some colonies of mice. We examined mouse strains to determine if, at the molecular level, there are different mechanisms of mammary tumor induction in high and low incidence strains.

Background and Rationale: For several decades MMTV has been thought to be involved in inducing spontaneous mammary cancer in some strains of inbred mice. Hybridization experiments performed in this laboratory and others have made it clear that several different MMTV variants may be involved in this process. The large number of copies of MMTV present in normal mouse cells have made it difficult to precisely define the mechanism by which MMTV transforms mammary epithelial cells. One can propose several mechanisms by which MMTV could transform normal mammary epithelium: differences in the nucleic acid sequence of the many endogenous and infectious MMTV variants; different genome locations of the integrated proviruses; specific expression of viral or adjacent cellular sequences; or variation in the target cells themselves may play a crucial role in cellular transformation. We have attempted to use restriction endonuclease analysis to develop molecular markers which distinguish among endogenous and infectious variants of MMTV, and thus provide us with a tool to begin to sort out the role different MMTVs play in the neoplastic transformation of murine mammary epithelial cells. We have attempted to correlate the presence of specific MMTV proviral variants with the incidence of mammary tumors in mouse strains with a low, moderate or high incidence of mammary cancer. We present data suggesting that several different mechanisms are involved in the transformation of mammary epithelium.

Major Findings: C3H/StWi mice have a low incidence of spontaneous mammary tumors. In spite of the fact that four distinct endogenous MMTV proviruses have been identified in this strain, only 7 out of 441 breeding females developed mammary tumors. To determine which, if any, of the endogenous proviruses was involved in inducing the mammary tumors, high molecular weight DNA from normal C3H/StWi tissue was digested with specific restriction endonucleases and the MMTV-specific DNA fragments identified by hybridization to MMTV-cDNA. Analysis of EcoR1 and Pst-1 restriction patterns allowed us to distinguish between the four endogenous proviruses. However, when the MMTV specific DNA fragments found in normal tissue was compared to those found in C3H/StWi spontaneous mammary tumors, the patterns were identical. The lack of additional MMTV-specific DNA fragments in mammary tumor tissue suggests that duplication of endogenous MMTV proviral information or infection with an exogenous MMTV variant is not involved in the transformation of normal mammary epithelium on C3H/StWi mice. Even when mammary tumors were induced in female mice with the chemical carcinogens DMBA or urethane, which in some cases increased the incidence of mammary tumors to 60%, no additional MMTV specific DNA fragments could be detected in mammary tumors when compared to normal tissues of the same animal. Indeed, the only C3H/StWi mammary tissues in which additional MMTV-specific DNA fragments were detected were those tumors arising in animals inoculated as newborns with the highly oncogenic MMTV(C3H). Consequently if MMTV is involved in the induction of spontaneous or chemically induced C3H/StWi mammary tumors it does so by another mechanism than the simple amplification of pre-existing endogenous MMTV genes. It should be noted that this is the first system in which spontaneous murine mammary tumors have been shown to arise without the presence of amplified MMTV proviral information.

Although the mammary tumor incidence in most strains of BALB/c mice is low, over 50% of the breeding females of the BALB/cV strain develop mammary tumors by the age of 10 months. To determine if any alteration in the MMTV proviral information of these mice was involved in this dramatic increase in tumor incidence, we examined the MMTV proviral DNA content of both normal and tumor tissue of BALB/cV mice. In addition to endogenous MMTV-specific bands found in all organs of BALB/cV mice, we identified two additional MMTV- specific restriction fragments in DNA from BALB/cV mammary tumors. These fragments appeared to contain the genetic information for an infectious MMTV provirus that could be distinguished from any of the endogenous BALB/cV proviruses. To date the additional MMTV proviral information found in mammary tumors of BALB/cV mice can be distinguished from any endogenous or exogenous MMTV variant reported thus far. In fact, we have been able to isolate an infectious virus from the BALB/cV strain of mice. This new MMTV variant has been shown to be oncogenic for BALB/c mice and also immunologically distinguishable (using monoclonal antibodies) from other infectious variants of MMTV. Thus it appears that the increased incidence of mammary tumors arising in BALB/cV mice is due to infection by a moderately oncogenic MMTV variant carried in this colony.

The GR strain of mice are interesting in that over 95% of breeding females develop mammary cancer before 1 year of age. Traditionally the MMTV has been thought to be involved in the induction of mammary tumors in GR mice. However, the mechanism by which MMTV might induce mammary cancer in the strain is unclear since both liquid hybridization studies and restriction endonuclease analysis has identified five endogenous MMTV proviruses in normal GR tissues. To identify which endogenous GR provirus(es) are involved in the induction of mammary cancer, crosses were set up between GR mice and C57BL mice in which the incidence of mammary cancer is 0%.

In BC1 females [C57Blx(C57BlxGR)] mammary tumor incidence segregated as a single Mendelian unit in that 50% of the animals developed mammary cancer. We have developed three molecular markers for a single endogenous GR provirus which also segregates as a single Mendelian unit and with the occurrence of mammary cancer in these mice. The markers are (1) MMTV-specific Sac 1 restriction fragments of 6.2, [illegible], and 0.9 Kbp, (2) MMTV-specific Bgl II restriction fragment of 4.3 Kbp and (3) a subset of nucleic acid sequences of the RNA genome of the highly oncogenic MMTV(C3H) virus called tumor-associated sequences. Thus we have identified a single endogenous GR provirus which causes the induction of mammary cancer in GR mice.

In summary, we have identified at least three mechanisms by which mammary tumors arise in mice. In the low tumor incidence C3H/StWi system there is no obvious involvement of MMTV either in spontaneous or chemically induced tumors, although a less obvious involvement, such as selective expression of specific viral or adjacent cellular sequences, has not been ruled out. In the moderate mammary tumor incidence strain, BALB/cV, a new infectious MMTV variant appears to be involved in the induction of mammary tumors. Finally, in the high mammary tumor incidence GR strain, we have identified a single endogenous GR provirus which seems to be involved in the high incidence of early occurring mammary tumors.

Significance to Biomedical Research and Proposed Course: It is now apparent that there are a wide variety of variants of the mouse mammary tumor virus differing in their nucleic acid sequence, antigenic specificity, site of integration, biological activity and mode of transmission. The large number of different MMTV variants thus far reported complicates the task of precisely defining which variant is actually involved in tumorigenesis in a given population. However, restriction endonuclease analysis and the Southern blot technique now allow us to distinguish between most of the endogenous and infectious MMTV proviruses. We have the potential to assign specific biological activities (such as virus production and neoplastic transformation) to specific genetic loci, such as the newly acquired MMTV proviral information in BALB/cV mammary tumors or the gene endogenous to GR mice. We now can attempt to define at a molecular level, the involvement of specific MMTV variants in the transformation process in both inbred and naturally occurring feral populations.

Publications:

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Drohan, W., Teramoto, Y. A., Medina, D., and Schlom, J.: Isolation and characterization of a new MMTV variant from BALB/c mice. Virology (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04996-04 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Murine Mammary Tumor-Associated Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
Others:	Jeffrey Schlom	Section Chief	LCMB	NCI
	William Drohan	Sr. Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)

Michigan Cancer Foundation, Detroit, Michigan
Baylor College of Medicine, Houston, Texas

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of these studies is to define mouse mammary tumor virus (MMTV) gene expression as a function of mammary tumorigenesis in low and moderate incidence mouse strains. Analyses by specific competition radioimmunoassay demonstrated that many of these mammary tissues contained large amounts of the major MMTV core protein (p28), but no detectable MMTV envelope glycoprotein (gp52). Such noncoordinate MMTV genome expression was found in primary and transplanted mammary tumors, and in preneoplastic mammary tissues of BALB/c mice, as well as in normal mammary tissue of Swiss albino mice. These mammary tissues exemplify a naturally occurring animal model system for studying noncoordinate expression of MMTV gene products. A new MMTV has also been isolated from mice of a subline of the BALB/c mouse strain. Monoclonal antibodies directed against MMTV proteins were used to clearly distinguish MMTV(BALB/c) from all other known MMTVs. These immunological and other molecular studies thus define MMTV(BALB/c) as a novel MMTV variant.

Project Description

Objectives and Historical Background: Numerous mouse strains are used as animal models for mammary tumorigenesis. Strains that have a low spontaneous mammary tumor incidence, such as BALB/c, are particularly interesting since they are highly susceptible to mammary tumor induction by a variety of agents. BALB/c mice experimentally infected with mouse mammary tumor viruses (MMTVs) readily express virus in their milk; resulting mammary tumors also contain MMTV virions and demonstrate the expression of MMTV antigens in ratios comparable to those found in intact MMTV. Mammary tumors induced in BALB/c animals by chemical carcinogens, on the other hand, have previously shown no expression of MMTV virions or MMTV antigens. In some instances, however, partial or complete MMTV expression has been observed in some BALB/c mammary tissues. Several recent studies, furthermore, have shown that both MMTV proviral DNA and relatively high levels of MMTV-specific RNA can be found in BALB/c mammary tissues; the higher levels of RNA were observed following hormonal stimulation or chemical and/or physical carcinogen treatment.

Previous studies have demonstrated that both preneoplastic hyperplastic alveolar nodule (HAN) outgrowth lines and mammary tumors derived from them in BALB/c mice contain significant levels of MMTV-specific RNA. However, mammary tumors arising from a specific HAN line (D2) have been routinely negative for MMTV B-particles by electron microscopy. Highly concentrated supernatant fluids of primary cultures of D2 mammary tumor cells also showed no evidence of representative MMTV virions by radioimmunoassay or by radioactive tracer studies.

A more thorough analysis of the murine mammary tumor systems has been facilitated by the recent development of both type-specific and group-specific competitive radioimmunoassays (RIAs) for the MMTV 28,000 MW (p28) major internal protein and both the major envelope glycoproteins of molecular weights 52,000 (gp52) and 36,000 (gp36). gp52 and gp36 molecules have been shown to be distinct polypeptides by both tryptic peptide mapping and radioimmunoassay studies. Since evidence has been presented for independent polypeptide chain initiation of MMTV glycoproteins and non-glycoproteins, the simultaneous use of these RIAs for both nonglycoproteins and glycoproteins may prove particularly useful in delineating quantitative differences in the expression of viral gene products.

It is the purpose of these studies to define the association between MMTV gene products and mammary tumorigenesis in spontaneously occurring and hormonally or chemically induced mammary tumors of low and moderate incidence mouse strains.

Major Findings:

I. Noncoordinate Expression of MMTV Gene Products. To examine the intracellular expression of MMTV antigens in BALB/c D2 mammary tumors, MMTV group-specific RIAs for the major core protein (p28) and for the major envelope glycoproteins (gp52 and gp36) were employed. Each of the group-specific RIAs has previously been shown to be highly specific for the individual MMTV component being monitored. Extracts of spontaneous mammary tumors of MMTV-positive C3H mice showed significant competition in all three

RIAs. The addition of cell-free extracts of D2 mammary tumors to each RIA showed the extracts to contain p28 antigen without MMTV gp52 or MMTV gp36. Approximately 0.04% of the total protein the the D2 mammary tumor extract was MMTV p28 related; less than 0.001% of total protein, however, was related to either of the MMTV glycoprotein antigens. The D2 mammary tumor extracts were also assayed in gp52 RIAs using anti-MMTV which reacts with the pre-gp70 polyprotein precursor to MMTV gp52 and with anti-MMTV gp52 which reacts with deglycosylated gp52. No competition by the D2 mammary tumor extract was observed in either of these RIAs while MMTV competed completely.

Several different transplant generations of the D2 mammary tumor in BALB/c mice have been assayed for MMTV antigens. In all the tumor extracts examined, noncoordinate expression of MMTV p28 versus MMTV gp52 was found.

Studies were undertaken to determine if this phenomenon of noncoordinate expression was observed in other murine mammary tissues, or if it was a unique property of BALB/c D2 mammary tumors. Several different types of mammary tissues were assayed for their relative content of MMTV p28, gp52, and gp36. Extracts of primary mammary adenocarcinomas arising in BALB/c(MCF) mice after hormonal stimulation competed completely in the MMTV p28 RIA; the amount of p28 present in these tumors was the same as that found in a spontaneous tumor arising in MMTV-infected BALB/cfC3H mice. In contrast to the BALB/cfC3H tumors which competed completely in both of the glycoprotein RIAs, the BALB/c(MCF) tumors showed no significant competition in either the gp52 or gp36 RIAs. Mammary adenoacanthomas also arise after hormonal stimulation in some BALB/c mice. These adenoacanthomas did not express any of the three MMTV antigens being assayed. Normal midpregnant mammary glands from BALB/c mice, both from MCF and BCM colonies, also failed to show expression of MMTV antigens. Benign dysplasias, which did not develop into tumors at a rate higher than the normal mammary gland, showed no expression of MMTV antigens.

Noncoordinate expression of MMTV antigens was not restricted to the BALB/c strain. A laboratory strain of Swiss albino mice carrying the milk-borne MMTV has been previously described. The milk-borne MMTV can be removed from this strain by foster nursing late embryos on MMTV-negative C57BL mice. Normal mammary glands from both the "MMTV-positive" Swiss mice and the "MMTV-negative", foster nursed, Swiss mice were assayed for MMTV antigens. Whereas the MMTV-positive mammary glands showed significant quantities of MMTV p28, gp52, and gp36, mammary glands from foster nursed Swiss mice were found to contain MMTV p28, but no MMTV gp52 and little, if any, MMTV gp36. It appears that noncoordinate expression of MMTV antigens is therefore not restricted to D2 mammary tumors or indeed BALB/c mice, and may be a more common phenomenon in murine mammary tissues than has previously been conceived.

II. Hormones, Chemicals and Proviral Gene Expression as Contributing Factors During Mammary Carcinogenesis in C3H/StWi Mice. C3H/StWi mice have spontaneously lost their exogenous MMTV(MMTV-S) and have become a low mammary cancer subline. They do not ordinarily express their endogenous MMTV provirus as virions. Virgin C3H/StWi females were exposed to chemical carcinogens, dimethylbenzanthracene and urethane in the presence or absence of chronic hormonal stimulation of the mammary gland by pituitary isografts. By 10 months of age, mammary tumors developed in 40% of the females given DMBA, and in 59% of those given DMBA and carrying pituitary isografts. With urethane treatment alone, 14% of the mice developed mammary cancer during a 12-month period; however, 74% of the mice bore mammary tumors when pituitary isografts were present. None of the females given pituitary isografts alone developed mammary cancer during the experimental period. Mammary tumors from each group were evaluated by radioimmune competition assay, immunoperoxidase and electron microscopy to determine the extent of endogenous MMTV gene expression. In addition, the mammary glands of some of the non-tumor-bearing mice were studied by whole mount and by histology to determine the type, extent, and number of mammary dysplasias present in each group. In general, there was no correlation between tumor incidence and the presence of mammary tumor virus antigens. Of 32 mammary tumors tested in all groups, 10 were positive at low levels for MMTV antigens. In the positive tumors, the internal MMTV gag gene antigen, p28, was prevalent. Immunoperoxidase studies on these same tumors gave quantitatively similar results. It appears from these observations that chemical carcinogenesis of C3H/StWi mouse mammary glands does not require or even favor the complete expression of endogenous MMTV genes. We conclude that C3H/StWi mice are susceptible to DMBA and urethane induction of mammary cancer and to an extent this process is positively influenced by the presence of hormonal stimulation.

III. Isolation and Characterization of a Novel MMTV from BALB/c Mice. A novel mouse mammary tumor virus (MMTV) variant has been isolated from mice of a subline (1293) of the BALB/cCrl Med mouse strain. Whereas breeding females of the parent BALB/cCrl Med colony have a mammary tumor incidence of 1%, 47% of the breeding females of this BALB/cCrl Med subline develop mammary tumors before 10 months of age. Foster nursing experiments demonstrated this virus, termed MMTV(BALB/c), was transmitted only by milk. The novel MMTV variant isolate was shown to be immunologically related to, but distinct, from the MMTV variants of C3H, GR, and RIII mice by a series of competition radioimmunoassays for the MMTV 28,000d major core protein (p28), and the 52,000d (gp52) and 36,000d (gp36) major envelope glycoproteins. Monoclonal antibodies directed against MMTV gp36 were also used to clearly distinguish MMTV(BALB/c) from MMTV(C3H), MMTV(RIII), MMTV(GR), MMTV(C3HfC57BL) and MMTV(A). MMTV -specific proviral DNA content of mammary adenocarcinomas arising in the BALB/cCrl Med subline was examined with restriction endonucleases and the Southern blot technique, and compared to the MMTV proviral DNA content of BALB/CAnDe mammary tumors. The virus arising from these latter tumors has been termed MMTV(O). Analysis of Eco RI digests of high molecular weight DNA from both types of mammary tumors demonstrated additional MMTV-related proviral sequences when compared to the DNA of normal BALB/c tissues. The patterns generated with the restriction endonucleases Bgl II and Sac I distinguished the additional MMTV-specific proviral information in the mammary tumors of the BALB/cCrl Med subline from the proviral information in the BALB/cAnDe mammary tumors. These immunological and molecular studies thus define MMTV(BALB/c) as a novel MMTV variant.

Significance to Biomedical Research and Proposed Course: Mammary tumorigenesis in mice may involve at least two transformation steps: the transformation from normal to preneoplastic and the transformation from a preneoplastic to a neoplastic cell population. Various carcinogens may act singly or synergistically to effect both transformation steps. Recent reports have demonstrated that, even in low mammary tumor incidence mouse strains such as BALB/c which normally repress the production of complete MMTV virion, significant levels of MMTV-specific RNA can be detected in tissues from hormonal and physical and/or chemical carcinogen-treated mice. In the studies reported here, several different types of mammary tissues from the BALB/c strain have been analyzed by radioimmunoassays for three major MMTV structural polypeptides: p28, gp52, and gp36. Analyses of normal, preneoplastic, and neoplastic mammary tissues by RIAs indicate that noncoordinate MMTV antigen expression may be a rather common phenomenon. Our results favor the conclusion that enhanced endogenous MMTV gene expression is not overtly involved in chemically induced mouse mammary tumors with C3H/StWi mice of this strain. Hormonal stimulation of the gland, primarily via prolactin, seems to favor some viral protein accumulation but this seems rather limited in both normal and neoplastic mammary tissue. Our conclusion is that in C3H/StWi mice, endogenous MMTV expression may not be required for the maintenance of the transformed state and further that the precancerous lesions produced by chemical treatment are apparently unrelated to those recognized as being the result of viral involvement. These results define the numerous mechanisms that may exist in the genesis of mammary tumors of a given species and delineate the wide diversity of viral gene products and modes of expression of these products that may exist. We are currently extending these studies to delineate the role in mammary tumorigenesis, if any, of viral genes and gene products, in naturally occurring feral populations of the genus Mus.

Publications:

Colcher, D., Horan Hand, P., Teramoto, Y. A., Wunderlich, D., and Schlom, J.: Use of monoclonal antibodies to define the diversity of mammary tumor viral gene products in virions and mammary tumors of the genus Mus. Cancer Res. 41: 1451-1459, 1980.

Horan Hand, P., Teramoto, Y. A., Callahan, R., and Schlom, J.: Interspecies radioimmunoassays for the major internal protein of mammary tumor viruses. Virology 101: 61-71, 1980.

Schlom, J., Colcher, D., Drohan, W., Horan Hand, P., Howard, D., and Teramoto, Y. A.: Systematics of murine mammary tumor viruses. In Brennan, M., Rich, M. A., and McGrath, C. M. (Eds.): Breast Cancer Research: New Concepts in Etiology and Control. New York, Academic Press, Inc., 1980, pp. 149-171.

Schlom, J., Drohan, W., Teramoto, Y. A., Young, J. M., and Horan Hand, P.: Diversity of mammary tumor viral genes and gene products in rodent species. In Essex, M., Todaro, G., and Zurhausen, H. (Eds.): Cold Spring Harbor Conferences on Cell Proliferation - Viruses in Naturally Occurring Cancer. New York, Cold Spring Harbor Laboratory, 1980, pp. 1115-1132.

Teramoto, Y. A., Horan Hand, P., Callahan, R., and Schlom, J.: Detection of novel murine mammmary tumor viruses by interspecies immunoassays. J. Natl. Cancer Inst. 64: 967-975, 1980.

Teramoto, Y. A., Medina, D., McGrath, C., and Schlom, J.: Noncoordinate expression of murine mammary tumor virus gene products. Virology 107: 345-353, 1980.

Smith, G. H., Teramoto, Y. A., and Medina, D.: Hormones, chemicals and proviral gene expression as contributing factors during mammary carcinogenesis in C3H/StWi Mice. Int. J. Cancer 27: 81-86, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05056-03 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Molecular Studies of RNA Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Reddy	Visiting Scientist	LCMB	NCI
OTHERS:	S. Aaronson	Chief, LCMB	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	A. Srinivasan	Visiting Pellow	LCMB	NCI
	A. Habara	Visiting Fellow	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
	D. Swan	Expert	LCMB	NCI
	R. Balachandran	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Molecular organization of Moloney sarcoma virus was studied by nucleotide sequence analysis. The entire M-MSV including the terminal redundancies of this genome were sequenced. These studies revealed several important biochemical features of this viral genome which enables us to understand the transcription, translation and mechanisms of integration of this viral genome. 2. Abelson-MuLV genome in its integrated form was cloned in E. coli. Biochemical analysis was carried out to understand the structural organization of this viral genome.
3. Studies were carried out to understand cellular targets for in vivo transformation by two clonal replication competent type C viruses--Moloney and Rauscher MuLV. Moloney MuLV was found to induce T-cell tumors while Rauscher MuLV produced tumors of B-cell origin. The two leukemia viral genes have been molecularly cloned and recombinants between the two viruses using the cloned DNA molecules are currently being generated to understand the tissue specificity of these two viruses.

Project Description

PROJECT 1

Objective:

Nucleotide sequence analysis of M-MSV genome.

Major Findings:

Methods were standardized for nucleotide sequence analysis of DNA molecules. The three methods that were standardized are Sanger's dideoxy method, Maxam and Gilbert's chemical cleavage method and Matt and Smith's nick-translation method. Using a combination of these three techniques the nucleotide sequence analysis of M-MSV genome cloned in *E. coli* using λ phage Charon 21A vector by Tronick *et al*. was undertaken.

To the present time the entire genome of Moloney murine sarcoma virus has been sequenced. Nucleotide sequence analysis demonstrates that the 5817 bp viral genome has two large terminal repeats of 584 bases (LTRs) at both 5' and 3' ends of the proviral genome. The LTRs were found to contain promoter-like sequences as well as mRNA capping and polyadenylation signals. In addition, they possess an 11-base inverted terminal repeat at each end. Thus, the structure of M-MSV genome with a LTR at each end resembles that of prokaryotic transposable elements. In addition, the sequence analysis reveals that the viral genome has the coding potential for the entire Moloney murine leukemia virus (MuLV) *gag* gene. Both *pol* and *env* genes have suffered large deletions, accounting for the inability of the virus to synthesize either protein product. The transforming region of MSV contains a large open reading frame which encompasses its entire cellular insertion sequence. This open reading frame has initiation and termination points within helper viral sequences. Comparison of the nucleotide sequence of the MSV genome with the known type C viral *gag* gene order and available amino acid sequence data on *gag* gene structural proteins leads to the following conclusions: 1) Both amino-terminal and carboxy-terminal ends of the viral *gag* gene are processed. 2) p15, p12, p30, and p10 polypeptides are coded contiguously. 3) The polymerase is coded within the same reading frame as that of the *gag* gene product.

Comparison of the sequence data with that of Abelson murine leukemia virus shows the presence of certain "hot spots" in the type C RNA viral genome that promote recombinational events leading to the generation of sarcoma viral genome.

Proposed Course:

The sequence analysis of the M-MSV genome indicates the possibility of occurrence of a splicing event during the formation of *gag* and *src* messenger RNA molecules. To further understand the biogenesis of these mRNA molecules, the 5' ends of these RNAs will be sequenced and the exact location of splicing will be determined.

The sequence analysis allows us to predict the amino acid sequence of the transforming protein. Synthetic polypeptide will be generated from the predicted amino acid sequences which in turn will be used to prepare antisera for the transforming protein. These antisera will be used for further characterization of the transforming protein coded by the M-MSV genome.

PROJECT 2

Objectives:

To study the molecular mechanisms involved in the transformation of fibroblast lymphoid cells by Abelson murine leukemia virus.

Major Findings:

The integrated proviral genome of Abelson murine leukemia virus (A-MuLV) was molecularly cloned in *E. coli* using bacteriophage vector λgt Wes λB. A 7.8 kbp DNA fragment containing the integrated viral genome was obtained following Eco RI digestion of high molecular weight DNA of A-MuLV nonproducer cells and enrichment by RPC-5 chromatography and preparative gel electrophoresis. Recombinant DNA clones containing a 7.8 kbp Eco RI fragment were shown to contain the entire integrated A-MuLV genome with 5' and 3' ends flanked by 1.8 kbp and 0.2 kbp mink cellular DNA sequences, respectively. A physical map of the proviral genome was generated and revealed that restriction enzymes Hind III, Bam HI, Cla I, Sal I, Pvu I and Bst III each cleaved at a unique site in the viral genome.

Cloned A-MuLV DNA was shown to transform NIH/3T3 cells at reasonably high efficiency. Moreover, such transformants contained the rescuable A-MuLV genome. In order to localize the region of A-MuLV required for transformation, we measured the infectivity of the proviral genome following exposure to different restriction enzymes. A-MuLV DNA cleaved with Sac I, Bgl I, Pvu I, Bgl II and Pst I lost detectable biologic activity. In contrast, cleavage of a A-MuLV proviral DNA with Hind III, Bam HI, and Cla I did not impair transforming activity. To eliminate the possibility that transfecting activity was due to incomplete restriction, A-MuLV subgenomic DNA fragments were prepared and cloned in plasmid vector pBR322. Transfection with subgenomic A-MuLV DNA clones helped to further localize the region of the viral genome necessary for transformation.

To further understand the molecular organization of the host-derived sequences, the DNA sequences from the normal cell genome which cross-hybridize with "leuk" sequences were cloned. Preliminary studies with these clones suggest the possibility that A-MuLV genome is derived from two independent genes.

Proposed Course:

The complete nucleotide sequence analysis of A-MuLV genome will be carried out. The cellular counterpart of "leuk" sequences that have been cloned will be tested for transforming activity before and after the addition of LTR viral sequences. Also, the location of the cellular "leuk" sequences in a normal mouse chromosome will be mapped using somatic cell hybrids.

PROJECT 3

Objectives:

To study tissue specificity and mechanisms of leukemogenesis of replication-competent Moloney and Rauscher mouse leukemia viruses.

Major Findings:

Studies were carried out to understand the cellular targets for *in vivo* transformation by two clonal replication-competent type C viruses--Moloney and Rauscher MuLV. Moloney MuLV-induced tumors and lymphoma cell lines exhibited Thy-1 antigen in the absence of detectable Fc and C3 receptors, indicating their T-cell origin. However, Rauscher MuLV primary tumors and lymphoma cell lines of the same mouse strain invariably exhibited Fc receptors in the absence of Thy-1 antigen, suggesting tumors were of the B-lymphoid lineage. The pattern of immunoglobulin synthesis by these various cell lines was determined both by biosynthetic and radioimmunologic techniques. Rauscher MuLV lymphoma cell lines invariably expressed immunoglobulin heavy (μ) chain in the absence of detectable light (κ or λ) chains. All these findings establish that the target of neoplastic transformation in response to Rauscher MuLV is an immature cell within the B-lymphoid cell lineage. To investigate the basis for their cell specificity, we have molecularly cloned these viruses, analyzed their molecular organization and attempted to construct recombinant viruses for biologic testing. The physical map obtained for integrated Moloney MuLV DNA, molecularly cloned in its Hind III permuted form, was consistent with that previously published (Gilboa *et al*., 1979). We cloned the R-MuLV genome in its integrated form. Cellular DNA was isolated from a clonal line of NRK cells productively infected with R-MuLV and digested with Eco RI. This enzyme was previously shown not to cleave the unintegrated linear R-MuLV genome. R-MuLV-specific DNA was enriched by RPC-5 column chromatography and sucrose density gradient and used for cloning in lambda phage Charon 4A. The cloned molecule is approximately 14 kbp in length. It was shown to contain an approximate 9 kbp viral DNA as well as host flanking sequences. The cloned DNA-induced virus production in NIH/3T3 cells upon transfection. Restriction enzyme analysis showed a strong correlation between the maps of cloned R-MuLV DNA and unintegrated linear R-MuLV DNA. Detailed characterization of the cloned integrated R-MuLV DNA, specifically its restriction enzyme mapping, was carried out.

Proposed Course:

Attempts will be made to understand the mechanisms which are responsible for tissue specificity of these two viruses. Attempts also will be made to generate recombinants between Rauscher and Moloney MuLV using the cloned proviral genomes in an attempt to map the region within the viral genome responsible for tissue specificity.

Publications:

Reddy, E.P., Smith, M.J., Canaani, E., Robbins, K.C., Tronick, S.R., Zain, S., and Aaronson, S.A.: Nucleotide sequence analysis of the transforming region and large terminal redundancies of Moloney-murine sarcoma virus. Proc. Natl. Acad. Sci. USA 77: 5234-5338, 1980.

Aaronson, S.A., Barbacid, M., Dunn, C., and Reddy, E.P.: Genetic approaches toward elucidating the mechanisms of type C virus-induced leukemia. In Neth, R. (Ed.): Modern Trends in Human Leukemia, Vol. 4. Berlin/New York, Springer-Verlag, 1981, pp. 455-459.

Cremer, K., Reddy, E.P., and Aaronson, S.A.: Translational products of Moloney murine sarcoma virus RNA: Identification of proteins encoded by the MSV src gene. J. Virology. (In press)

Srinivasan, A., Reddy, E.P., and Aaronson, S.A.: Abelson murine leukemia virus: Molecular cloning of infectious integrated proviral DNA. Proc. Natl. Acad. Sci. USA. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05059-03 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Development of Assay for In Vitro Transformation by Murine Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Nagao	Visiting Fellow	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	J. Pierce	Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)
Teruko Ishizaka, Professor, Immunology Division, Johns Hopkins University;
Kenjiro Yokoro, Professor, INMB, Hiroshima University, Japan

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Culture techniques for bone marrow culture of normal hematopoietic cells have been established in the last two years in our laboratory. Using these techniques, non-tumorigenic bone marrow cells from W/W^v and Sl/Sl^d mice, which have a deficiency in the CFU-s or CFU-c compartment, have been maintained for more than 6 months. These mutant mice lack mast cells in skin and connective tissues. However, the established cell lines in vitro showed metachromasia by toluidine blue stain and had histamine and receptors for I_gE, strongly indicating that these mice have at least committed stem cells for basophil/mast cells. The cell lines also showed CFU-META in semi-solid culture. After infection by oncogenic type C viruses, virus producing basophil/mast cell lines from NFS/N mice have been maintained for more than 3 months. Examination of in vitro transformation in suspension culture systems is now in progress.

PHS-6040
(Rev. 2-81)

P[illegible] Description

Objectives:

1. To establish a method of l[illegible] culture of hematopoietic cells.

2. To examine optimal culture [illegible]ions to investigate differentiation of hematopoietic stem cell[illegible]

3. To examine the relationshi[illegible]en the differentiation stage of target cells and malignant [illegible]ormation by oncogenic type C RNA viruses.

Methods Employed:

Different culture systems are [illegible] to find the optimal conditions for long-term culture of hematopo[illegible]get cells. Differentiation inducers and inhibitors are examined t[illegible] the maturation of hematopoietic stem cells. Highly leukemogenic m[illegible]eukemia viruses have been isolated, and cloned viruses are used to in[illegible]atopoietic cells. Changes in differentiation markers of hematopoieti[illegible] are examined as well as changes in growth properties of these ce[illegible]*tro* and *in vivo*.

Major Findings:

1. Culture methods for maint[illegible] hematopoietic cells from spleen [illegible] bone marrow have been est[illegible]

2. Culture conditions such a[illegible]tration of growth factor(s) and serum affect the maturation of [illegible]/mast cells.

3. W/W^v and Sl/Sl^d mice lack[illegible]ing factors for basophil/mast cell[illegible] *in vivo*.

4. Basophil/mast cells are [illegible]e to infection by oncogenic type C RNA viruses.

Significance to Biomedical R[illegible] the Program of the Institute:

For understanding of viral l[illegible]esis, culture methods for propagation of normal lymphoid and myelo[illegible]s may be of great importance. Such techniques may make it possi[illegible] study the regulation of differentiation of normal hematopoietic cell[illegible] test for *in vitro* transformation by oncogenic viruses.

Proposed Course:

1. Examine the stages of he[illegible]c cell differentiation at which cells are susceptible to infec[illegible] variety of oncogenic type C RNA viruses.

2. Analyze the effect of leukemia viruses and other agents on normal hematopoietic cell differentiation, as well as their ability to cause malignant transformation.

3. Develop specific markers for differentiation of hematopoietic cells.

Publications:

Nagao, K., Yokoro, K., and Aaronson, S.A.: Continuous lines of basophil/mast cells derived from normal mouse bone marrow. Science 212: 333-335, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05060-03 LCMB

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of Oncogenic Expressions in Experimental Animal and Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.S. Rhim	Research Microbiologist	LCMB	NCI
Other:	R.J. Huebner	Head, Viral Immunology Section	LCMB	NCI
	K. Koh	Visiting Fellow	LCMB	NCI
	R. Trimmer	Bio. Lab. Tech.	LCMB	NCI
	G. Smith	Bio. Lab. Tech.	LCMB	NCI
	A. Scheib	Bio. Lab. Tech.	LCMB	NCI

COOPERATING UNITS (if any) S. Aaronson, LCMB, NCI; P. Arnstein, LCMB, NCI; D. Ablashi, LCMB, NCI; R. Adamson, LCHPH, NCI; D. Fish, FCRC; R. Gilden, FCRC; J. Cicmanec, Litton-Bionetics; M. Gardner & S. Rasheed, USC; J. Frankel, U. of Florida; S. Kalter & R. Heberling, S. W. Found. for Res. & Ed.; C. Graham, U. of Albany

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Viral Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to (1) establish and define a cell culture transformation system for identification of genetically defective individuals and people exposed to environmental carcinogens, (2) identify and rescue human and primate sarcoma (src) information, and (3) develop and test vaccines and other preventive measures to prevent and control cell transformation and cancers in animal and primate systems and ultimately in humans.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. To develop a cell culture transformation system for identification of individuals at high risk for early cancer.

2. To search for and rescue human and primate sarcoma (src) information from primate and human cancers.

3. To develop a vaccine and other immunopreventive measures to prevent and control transformation and cancer in animals and ultimately in humans.

Methods Employed:

Biological methods include cell and virus cloning, transformation focus, cell aggregation, soft agar, genome rescue and transfection assays. Biochemical methods include RNA-dependent DNA polymerase, radio-immunoprecipitation assay and I^{125} protein A assay.

Major Findings:

1. Human skin fibroblasts derived from cancer family syndromes, such as ACR and Gardner's, furnished and tested by Drs. L. Kopelovich and M.B. Gardner were tested for focus formation by Ki-MSV. The results confirmed the genetic differential susceptibility of cultured human skin fibroblasts to transformation by Ki-MSV. Thus tests for identification of high risk individuals in families carrying autosomal dominant genes for cancer were confirmed in three laboratory test systems. In order to establish further its accuracy, the coded skin samples received from Drs. E. Gardner and R. Moon are currently being tested for their susceptibility to Ki-MSV.

2. Increased transformation efficiency of Ad12-SV40 was observed in human skin cultures derived from high risk autosomal dominant cells. Transformation was evidenced by morphological alteration and presence of Ad12-SV40 "T" antigens by means of the complement-fixation test. These transformed cells contained low levels of virus. However, they formed large cell aggregates and colonies in soft agar and then became permanent lines. Characterization of established lines of Ad12-SV40 transformed skin fibroblasts is currently in progress.

3. Human skin fibroblasts derived from ACR individuals are sensitive to a chemical carcinogen. An MNNG-treated human skin (PF) line showed morphological alteration and formed large cell aggregates above an agar overlay and formed colonies in soft agar. "Transformed cells" were resistant to rechallenge of MNNG (1 μg/ml) and showed a prolonged life span compared to untreated cells. However, tumors were not produced in nude mice by subcutaneous inoculation. Attempts to produce tumors in nude mice by eye injection were not successful. Chemical carcinogens alone may not induce neoplastic transformation of fibroblasts from humans genetically predisposed to cancer. Neoplastic transformation of human skin cells by a chemical carcinogen apparently requires a multiple process.

4. Susceptibility of chimpanzee skin fibroblasts to viral transformation. Chimp skin cells can be readily transformed *in vitro* by RNA and DNA tumor viruses. Ki-MSV and Ki-MSV(BaEV) transformed chimp skin cells were found to be virus producers, whereas RSV transformed chimp skin cells were found to be nonproducers (NP). Ad12-SV40 transformed chimp skin cells contained Ad12-SV40 large tumor antigens and were found to be virus producers. One NP Ad12-SV40 transformed (WES) line was isolated. Tumors were produced when the NP cells were transplanted into nude mice. Ki-MSV(BaEV) transformed chimp skin cells produced tumors when the transformed cells were transplanted into nude mice. However, the tumors produced were murine tumors. Various clonal lines were established and characterization studies are in progress.

5. Induction of tumors in chimps by virus or chemical carcinogen. Attempts to induce tumor in chimps by inoculating Ki-MSV(BaEV), Ad12-SV40, or a chemical carcinogen (DENA) are in progress. Ki-MSV(BaEV) or Ad12-SV40 transformed chimp skin cells were also inoculated into chimps for tumor induction, but no tumors have been obtained to date.

6. Isolation and characterization of S^+L^- (sarcoma-positive, leukemia-negative) human osteosarcoma (HOS) cells induced by the feline leukemia virus pseudotype of Moloney sarcoma virus [M-MSV(FeLV)]. These clones were negative for infectious virus and type C virus particles; however, they contained a rescuable M-MSV genome and murine leukemia virus (MuLV)-specific antigens, such as the *gag* gene products p15, p12 and p30, but lacked the *env* gene protein gp70. S^+L^- clones were all tumorigenic in nude mice. The M-MSV was rescued by baboon type C virus (M-7). M-MSV(M-7) produced foci readily in human, NRK and NIH 3T3 mouse embryo cells. Human cells transformed by the M-MSV(M-7) were found to be virus producers, whereas M-MSV(M-7) transformed NRK and NIH 3T3 mouse embryo cells were found to be S^+L^- cells. Thus the Moloney MSV was able to infect human HOS cells with the production of S^+L^- cells having a transformed phenotype. These data indicated also that S^+L^- cells could be isolated from rat and mouse cells by means of simple M-MSV(M-7) virus infection and subcultivation.

7. Transformation of feline embryo cells by a chemical carcinogen. Repeat treatment of 7,12-dimethylbenz[a]anthracene (DMBA) induced *in vitro* transformation of feline embryo cells following clonal growth and selection. The DMBA treated cells had a number of transformation-associated markers: (1) altered morphology, (2) increased growth rate, (3) formation of larger cell aggregates and growth in this aggregate form above an agar base, and (4) formation of colonies in soft agar with high efficiency. However, no progressively growing tumors were produced when inoculated into nude mice. Feline cell transformation by a chemical carcinogen is characterized by extended preneoplastic stages *in vitro* and differs from the other mammalian cell models of carcinogenesis. The transformed lines were negative for feline oncornavirus-associated cell membrane antigen (FOCMA), a "transformation specific" antigen which is distinct from the virus structural proteins of feline leukemia virus.

8. Viral transformation of human epithelial cells. Since most human tumors are of epithelial origin, the importance of studying human epithelial cell carcinogenesis is well recognized. However, normal human epithelial cells are very difficult to culture. Therefore, there are few reports describing the viral transformation of human epithelial cells. Recently a new culture medium, NCTC 168, designed for human skin epithelial cells was developed by Dr. Sanford and her associates. A viral transformation study utilizing primary culture of human skin epithelial cells grown in NCTC 168 supplemented with horse serum has recently been initiated. Primary human epithelial cells infected with the Ad12-SV40 virus grew, differentiated and became established cell lines, whereas the infected cells did not grow. Various clonal lines were established and characterization studies are in progress.

Significance to Biomedical Research and the Program of the Institute:

Development of reproducible testing systems for identification of relative genetic susceptibilities to cancer is much needed in cancer diagnosis. Development of primate and human cell lines for rescue and identification of primate and human common tumor antigens will lead to eventual application to the development of protective vaccines against primate and human cancers. Development of a new model for the study of human epithelial cell carcinogenesis is important in understanding the process of neoplastic conversion in human epithelial cells.

Proposed Course:

1. To define further the Ki-MSV human skin transformation system and establish its validity and parameters as possible diagnostic indices for detection of hereditary cancer in man.

2. Characterize the established lines of Ad12-SV40 transformed human skin fibroblasts derived from genetically abnormal individuals.

3. The role of tumor promoter agent (TPA) as well as nontransforming retrovirus in MNNG transformation of a human skin cell system will be studied in order to develop a model for cancer promotion *in vitro.*

4. To develop chimpanzee tumors transplantable in chimpanzees to serve as allogeneic vaccines and syngeneic challenge.

5. To rescue *src* expressions specific for primate and human tumors. The following efforts will be pursued:

a. Induction of tumors in newborn chimps by tumor viruses or chemical carcinogens.

b. Isolation and characterization of variants from human and primate cells transformed by RNA tumor viruses or DNA tumor viruses.

c. Isolation and characterization of revertants from S^+L^- human HOS cells which may provide a potentially important model for the understanding of the mechanism involved in cellular gene expression and tumorigenicity.

d. Seek cocultivation of chimp tumor cells that have been transplanted in a natural host one, two, or three times, based on rescue of rat src sequences.

e. Attempt to demonstrate the transforming activity of human testicular tumor DNAs by transfection assay.

6. Further characterization of clonal lines of Ad12-SV40 transformed human epithelial cells. Attempt to transform human epithelial cells by a chemical or chemical plus virus.

7. Examine the protective effect against tumors of primates by infection with xenotropic type C viruses.

Publications

Rhim, J. S., Huebner, R. J., Arnstein, P., and Kopelvich, L.: Chemical transformation of cultured human skin fibroblasts devised from individuals with hereditary adenomatosis of the colon and rectum. Int. J. Cancer 26: 565-569, 1980.

Snyder, H. W., Phillips, K. J., Hardy, W. D., Zuckerman, E. E., Essex, M., Sliski, A. H., and Rhim, J. S. Isolation and characterization of proteins carrying the feline oncornavirus-associated cell-membrane antigen. Cold Spring Harbor Symp. on Quant. Biol. 44: 787-799, 1980.

Rhim, J. S., Trimmer, R., Arnstein, P., and Huebner, R. J.: Neoplastic transformation of chimpanzee cells induced by adenovirus type 12-simian virus 40 hybrid virus. Proc. Natl. Acad. Sci. 78: 313-317, 1981.

Rhim, J. S. Characterization of sarcoma-positive, leukemia-negative (S^+L^-) human cells induced by the feline leukemia virus pseudotype of Moloney sarcoma virus. Proc. Soc. Expt. Biol. Med., 1981 (In press).

Rhim, J. S., Arnstein, P., and Huebner, R. J.: Chemical transformation of cultured skin fibroblasts from humans genetically predisposed to cancer. In: Proceedings of the Fourth International Symposium on Prevention and Detection of Cancer. New York and Basel, Marcel Dekker, 1981 (In press).

Rhim, J. S., Chen, A., and Essex, M.: Characterization of clones of tumorigenic feline cells transformed by a chemical carcinogen. Int. J. Cancer, 1981 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05061-03 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Functional Organization of Abelson Murine Leukemia Virus Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Srinivasan	Visiting Fellow	LCMB	NCI
Other:	E. P. Reddy	Visiting Scientist	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI
	J. Pierce	Staff Fellow	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
	S. Tronick	Research Microbiologist	LCMB	NCI
	A. Eva-Varesio	Visiting Associate	LCMB	NCI

COOPERATING UNITS (if any)

R. Gallo, Chief, LTCB, NCI

LAB/BRANCH
Laboratory of Cellular and Molecular Biology, Carcinogenesis Intramural Program

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an effort to understand the structural and functional organization of A-MuLV, integrated proviral genome was molecularly cloned in *E. coli* using bacteriophage vector λgtWES·λB. A physical map of the proviral genome was generated and revealed that restriction enzymes Hind III, Bam HI, Cla I, Sal I, Pva I and Bst EII each cleaved at a unique site in the viral genome. Cloned A-MuLV proviral DNA was shown to transform NIH/3T3 cells. Moreover, such transformants contained the rescuable A-MuLV genome. In order to localize the region of A-MuLV required for transformation, the biological activity of the proviral genome following exposure to different restriction enzymes was tested. A-MuLV DNA cleaved with Sac I, Bgl I, Pvu I, Bgl II and Pst I lost detectable biologic activity. In contrast, cleavage of A-MuLV DNA with Hind III, Bam HI, Sal I and Cla I did not impair transformation ability. Subgenomic fragments of A-MuLV cloned in plasmid vector pBR322 confirmed the above findings and helped to map the region needed for transforming function of A-MuLV.

Project Description

Objectives:

To study the functional organization of Abelson murine leukemia virus.

Methods Employed:

Molecular cloning techniques, transfection assay, immunoprecipitation and SDS-PAGE.

Major Findings:

The integrated proviral genome of Abelson murine leukemia virus was molecularly cloned in *E. coli* using bacteriophage vector λgtWES·λB. A 7.8 kbp DNA fragment containing the integrated viral genome was obtained following Eco RI digestion of high molecular weight DNA of A-MuLV transformed nonproducer cells and enrichment by RPC-5 chromatography and preparative gel electrophoresis. Recombinant DNA clones containing a 7.8 kbp Eco RI fragment were shown to contain the integrated A-MuLV genome with 5' and 3' ends flanked by 1.8 kbp and 0.2 kbp mink cellular DNA sequences, respectively. The relationship of A-MuLV to its parental genome M-MuLV was studied by heteroduplex analysis. Such studies showed that A-MuLV contains regions of homology with M-MuLV at either end of its genome and the central portion is unique to A-MuLV. The extent of homology is 1.7 kbp and 0.7 kbp at 5' and 3' ends, respectively.

Cloned A-MuLV DNA transforms NIH/3T3 cells upon transfection. Moreover, focus-forming virus could be rescued from transformed nonproducer cells upon superinfection with a type C helper virus. In order to localize the region of A-MuLV required for transforming function, subgenomic fragments of A-MuLV were generated with Bam HI, Sal I, Hind III, Cla I and Pvu I and cloned in plasmid vector pBR322. The biological activity of the subgenomic clones were at a lower efficiency than genomic DNA. The subgenomic A-MuLV DNA clones which lacked the 3' LTR were biologically active, whereas the clones which lacked the 5' LTR and the adjoining sequences (downstream of LTR) showed no transforming activity. By this method, it has been possible to suggest that a stretch of 2 kbp DNA sequence from 3' end of viral genome is not required for transformation.

A-MuLV genome has been shown to code for expression of Mr 120,000 protein (p120). There is evidence that p120 and the associated protein kinase activity is required for transformation. This polyprotein was invariably detected by immunoprecipitation analysis of transformants induced by cloned proviral DNA. A-MuLV-specific viral proteins are being studied in the cells transformed by subgenomic A-MuLV DNA.

Significance to Biomedical Research and the Program of the Institute:

A-MuLV, isolated by passage of M-MuLV in a corticosteroid treated BALB/c mouse, appears to have arisen in nature by a mechanism involving genetic recombination between M-MuLV and mouse cell DNA sequences. Functional analysis of the A-MuLV genome revealed that the M-MuLV-specific sequence acts as a carrier for expression of a normal mouse gene acquired by A-MuLV. Detailed analysis of the A-MuLV unique sequences would be of help to approach the questions concerning putative transforming genes of human neoplasia.

Proposed Course:

1. The large terminal repeat (LTR) of A-MuLV is quite similar to the LTR of M-MuLV. Sequence analysis showed that the LTR contains putative promoter sites. However, neither the initiation site for transcription nor the location of the promoter has been determined. An attempt will be made to study the functional potential of LTR.

2. Studies will be carried out towards elucidating the formation of the transforming gene of A-MuLV.

Publications:

Srinivasan, A., Reddy, E.P., and Aaronson, S.A.: Abelson murine leukemia virus: molecular cloning of infectious integrated proviral DNA. Proc. Natl. Acad. Sci. USA 77: 2077-2081, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05062-03 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Expression of Retrovirus Transforming Genes and their Cellular Analogues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Eva-Varesio	Visiting Associate	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	K.C. Robbins	Expert	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purposes of this project are: 1) detection and characterization of the trancriptional products of the Moloney murine sarcoma virus (M-MSV) src gene and their involvement in transformation; 2) investigation of the possible role of retroviral transforming gene analogues in human neoplasia.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1) To characterize the transcriptional products of M-MSV in order to determine their genetic organization and their involvement in transformation.

2) To investigate whether DNA analogues of retroviral transforming genes are actively transcribed in human cancer cells in order to analyze the possible role of these genes in human neoplasia.

Major Findings:

1. We have analyzed cells transformed by wild type and deletion mutants of M-MSV. All these cell lines showed as major viral transcripts genomic-length polyadenylated RNAs. Other minor transcripts were identified, one of which was present in all the cell lines tested. By hybridization to ^{32}P-labeled DNA fragments of M-MSV genome, this mRNA was found to be comprised almost in its entirety of MSV src gene sequences. As such it appears to be a good candidate for the mRNA involved in virus-mediated cellular transformation.

2. RNAs extracted from a large number of human tumor derived cell lines were analyzed in hybridization experiments with DNA probes specific for the cell-derived sequences of several transforming retroviruses. Four of these probes recognized related transcripts in some of the cell lines tested. Thus, viral src gene analogues in human DNA are expressed in a variety of different tumors.

Significance to Biomedical Research and the Program of the Institute:

Since many transforming RNA tumor viruses are known to cause sarcomas and hematopoietic malignancies through the expression of certain genes derived from a set of well-conserved cellular genes, it is of interest to investigate whether and how these genes are specifically expressed in naturally occurring and virus-mediated malignancies.

Proposed Course:

1. To extend our study on M-MSV src transcripts to mouse cell lines transformed by molecularly cloned M-MSV DNA or subcloned DNA fragments.

2. To extend the study of expression of virus transforming gene analogues in human tumor cells in order to better define the specificity of them.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05063-03 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on Epstein-Barr Virus and Herpesvirus Saimiri

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Ablashi	Coordinator of DNA Virus Studies	LCMB	NCI
OTHERS:	P. Levine	Chief, Clinical Studies Section	LVC	NCI
	S.K. Sundar	Visiting Fellow	LVC	NCI
	J. Dahlberg	Research Microbiologist	LCMB	NCI
	A. M. Faggioni	Visiting Fellow	LCMB	NCI
	P. Gerber	Director, Viral Genetics Branch	DV	BB
	I. Magrath	Visiting Scientist	DCT	NCI

COOPERATING UNITS (if any) R. Krueger, Pathology Inst., Univ. of Cologne, West Germany; G. Pearson, Mayo Clinic, Rochester, Minn.; A. Bouguermouh, Inst. Pasteur of Algeria, Algeria; U. Prasad, Univ. of Malaya, Malaysia.

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Low passages of the P_3HR-1 cell line, derived from nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL), were found to contain cell populations still capable of producing transforming virus, contrary to prior reports, which were stimulated through biological manipulation. These findings were supported by reactivity of monoclonal antibody directed against transforming EBV membrane glycoprotein, which stained cells propagated at 34° but not those propagated at 37°C. Monoclonal antibody prepared against partially purified herpesvirus saimiri (HVS) was specific for HVS late antigen, failed to react with early and membrane antigen-producing cells, did not neutralize HVS, and did not react with either strain of herpesvirus ateles. IgA to EBV capsid antigen and antibody-dependent cellular cytotoxicity in NPC patients correlated with prognosis and treatment. Four anti-inflammatory agents and one protease produced variable effects on EBV early antigen expression; three of the anti-inflammatory agents inhibited early antigen induced by TPA, whereas no inhibition of early antigen induced by virus was seen, suggesting that TPA and virus induction of early antigen are mediated by different mechanisms.

Project Description

Objectives:

A. To study the herpesviruses associated with NPC and BL by investigating the strain differences of EBV, and by exploring the role of co-factors, *i.e.*, TPA, which may shed light on the multifactorial and multisteps etiology of these tumors. Secondly to use EBV-related serology in long-term prognosis in NPC patients, especially those with undifferentiated NPC. Thirdly to ultilize HVS immunovirology in understanding the role of primate oncogenic herpesvirus in cancers.

B. To initiate and participate in an international collaborative research program on herpesviruses of primates which includes my laboratory and other national and international institutions and organizations.

Method Employed:

A. Primary cell cultures and continuous cell lines of human and animal origin were used for virus isolation, biological and biochemical assays, and for *in vivo* experimentation. Standard biochemical and immunological procedures were applied. Human subjects were involved in the research projects by obtaining sera from patients with various cancers of interest, healthy donors, and individuals working in the laboratory with oncogenic herpesviruses.

The interaction of TPA (12-O-tetradecanoyl-phorbol-13-acetate), anti-inflammatory agents (dexamethasone, prednisolone, hydrocortisone, cortisone) and N-α-P-Tosyl L-Lysine Chloromethyl Ketone HCl (TLCK) with EBV antigens were investigated using EBV nonproducer Raji cells. N-methyl-N-nitrosoguanidine (MNNG) was employed in studying the interaction of EBV-induced EA.

Monoclonal antibody to HVS and transforming EBV and EBV (B95-8) membrane glycoprotein, i.e., 320K were prepared by the hybridoma technology. Athymic NIH Swiss mice (Nu/Nu) were used for tumorigenicity and testing for specific transfer factor.

Major Findings

A. (1) In collaboration with Drs. Gerber and Magrath, recent passages of P_3HR-1 cells obtained from five different sources showing HLA A3, B17 and BW35 were tested for production of transforming and lytic (nontransforming) EBV by growing the cells at 37°C and 34°C for virus preparation or by growing the cells at 37°C and aging at 32°C or 35°C. The virus prepared from these preparations (32°C, 34°C, and 35°C) contained 1-4 logs of transforming EBV as well as $\geq$ 4 logs of lytic EBV, suggesting that P_3HR-1 cells can be stimulated to produce tranforming EBV by biological manipulations. The virus preparation from cells at 37°C were inconsistent in transforming human cord blood lymphocytes, suggesting that either cells capable of producing tranforming virus are shut off or that amounts of virus produced are too low for transformation.

The transformed human cord blo[illegible] contained EBV nuclear antigen, and EBV-DNA viral functions were compl[illegible] ressed, but the transforming genome could be rescued by superinfection w[illegible] eration of lytic P_3HR-1 EBV. Transformed cells after 7 or 8 in v[illegible] ges induced undifferentiated lymphoma in nude mice previously treate[illegible] -lymphocyte serum.

The P_3HR-1 cells carried eithe[illegible] ver aged at 32°C and 35°C revealed the presence of 10-15% cells which [illegible] the immunofluorescence test (IF) with monoclonal antibody directed a[illegible] 320K glycoprotein of transforming (B95-8 strain) EBV. The reactivity w[illegible] in the early stages and full rings in late stages of cell aging.

(2) In collaboration with Drs[illegible] and Dahlberg, monoclonal antibodies to B95-8 EBV (16BA11, 160G3), to [illegible] purified HVS (19CH9), and two membrane glycoproteins of transforming [illegible] 6 and B10) were characterized. The 16BA11, an IgM antibody, reac[illegible] uorofluorescence (IF) with cells producing tranforming EBV, whereas 16DG3 [illegible] antibody, failed to show any reactivity. Antibody to 320K glycoprotein [illegible] ed as IgG2b, reacted in the IF test with cells producing AG-876, B95-8 [illegible] EBV. All these cell lines produce varying degrees of transformi[illegible] s antibody did not react with cells producing lytic P_3HR-1 virus. [illegible] 10 (IgM class) showed the reactivity since it stained cells (P_3HR-[illegible] both types of EBV.

Monoclonal antibody 19CH9, be[illegible] gG, was specific to HVS late antigen producing cells in the IF ass[illegible] ed to react with HVS early and membrane antigen producing ce[illegible] s failed to neutralize HVS. Moreover, it lacked cross reactivity wi[illegible] ains of Herpesvirus ateles (73 and 810 strains). Interestingly, thi[illegible] nly stained [illegible] cells by immunofluorescence in compari[illegible] staining of some cells with anti-HVS serum obtained from owl monke[illegible] HVS-induced lymphoma.

(3) In collaboration with Drs[illegible] Levine and Faggioni, four anti-inflammatory agents (dexameth[illegible] isolone, hydrocortisone, cortisone) and a protease N-α-P-Tosyl L-[illegible] omethyl Ketone HCl (TLCK) were found to inhibit TPA induced EBV-EA [illegible] cells in varying degrees, but TLCK did not have any significant effe[illegible] tion, EBV-induced EA was not inhibited by any of the steroids and pr[illegible] emoval of these agents resulted in partial recovery of EA in TPA[illegible] cells. Since TPA did not induce DNA synthesis in Raji cells, the [illegible] tion by TPA is independent of the tumor promoter's ability to s[illegible] synthesis in other cell systems.

(4) Treatment of Raji cells [illegible] prior to induction of EA by superinfection with lytic EBV from [illegible] ted in 10-100 fold higher EA-positive cells than in the absence of [illegible] er, TPA induced EA in Raji, NC-37 and Walker lymphoblastoid cells c[illegible] V genome was not influenced by MNNG. Moreover, those EA producing [illegible] t produce EBV virus capsid antigen (VCA). The data indicated th[illegible] as a potent enhancer of EBV expression, suggesting it cou[illegible] n a practical immunological ass[illegible] as well as for studying the rol[illegible] rs involved in development of [illegible] and BL.

(5) In collaboration with Dr. Whitman, three purified human interferon preparations were tested against human herpesviruses (Herpes simplex-1, Varicella-Zoster and EBV) and human rhabdomyosarcoma tumor cells. All three interferons (leukocyte, lymphoblastoid and fibroblast) with specific activities of approximately 1.0×10^6 μ/mg protein, drastically restricted the replication of Varicella Zoster virus, Herpes simplex type 1 virus and also inhibited the growth of rhabdomyosarcoma tumor cells in doses of 300-3,000 μ/ml. Some doses of purified lymphoblastoid interferon had some significant antiviral effect on the AG-876 strain of EBV. Thus these interferon preparations may be useful in investigating the growth inhibiting property of lymphoblastoid transformed cell by EBV or cell lines established from BL and NPC tumors.

In collaboration with Drs. Pearson, Krueger, and Levine, sera from eleven patients with NPC of the undifferentiated and non-keratinizing types from the Institute of Pathology, University of Cologne, West Germany were followed for the last three years for prognosis to treatment using IgA antibody to EBV VCA and antibody to ADCC. The IgA antibody to VCA and IgG antibody to EA were rarely detected in the non-NPC sera. In two cases the follow-up sera showed that IgA antibody dropped significantly and was accompanied by remission. The titers to ADCC were higher in 5 cases, 4 of which did not suffer recurrences. The low ADCC titers, at least in 3 patients, indicated no tumor regression. In the majority of cases, sera with higher ADCC titers contained lower anti-IgA titers, suggesting that poor prognosis may be due to blocking activity of IgA antibody.

In collaboration with Drs. Pearson, Prasad, Krueger and Bouguermouh, serum antibody titers to several EBV antigens were investigated in NPC patients from intermediate incidence areas (Algeria, Tunisia, and Malaysia) and from low incidence areas (West Germany and USA). Geometric mean titers to VCA-IgA, VCA-IgG, EA-IgG were higher among patients from intermediate risk countries compared to those from low risk countries, but the only statistically significant difference was for VCA-IgG. The influence of sex and age of the patients and the stage of the disease on the antibody titers showed no significant difference except in one patient. Sera tested from 42 NPC patients showed a positive correlation between VCA-IgA and EA-IgA antibodies, emphasizing that the initial diagnosis of NPC should include detection of IgA antibodies to both VCA and EA.

Transfer factor (TFd) has been used with some success for treatment of patients with various malignancies. Due to increasing evidence of EBV involvement in NPC (work done in collaboration with Drs. Pizza, Viza, and Levine) specific *in vitro* TFdL using lymphoblastoid cell line LDV/7 was produced. This TFdL was directed against surface antigens of Raji cells superinfected with lytic P_3HR-1 EB virus and from several other sources: humans with infectious mononucleosis (IM) or NPC; guinea pigs immunized with B95-8 EBV and tumor bearing nude mice injected with P_3HR-1 cells. Transfer of immune reactivity against EBV-associated membrane antigens (MA) was achieved in rhesus monkeys injected with TFdL originally derived from patients with NPC. Thus it was possible to produce *in vitro* large amounts of active TFdL with standardized activity which produced more encouraging results than observed in previous investigations with IM-derived transfer factor in NPC patients. Such TFdL could be used for clinical trails in NPC patients alone or in conjunction with other therapy.

Previous investigations to study cellular immunity to tumor and/or virus-related antigens in NPC patients have been hampered by non-availability of standardized antigens which can be utilized over a period of years and provide comparability of assays. We have been able to preserve membrane antigens of EBV-infected cells with modified formalin fixative. Using these fixed cells as a source of antigen, normal donors seropositive to EBV, and NPC patients were shown to have cell-mediated immunity (CMI) to EBV-MA by both leukocyte migration inhibition and lymphocyte stimulation assays. Thus, it appears promising to initiate longitudinal studies to assess the role of CMI in the monitoring and treatment of patients with NPC.

B. In collaboration with Drs. Krueger and Prof. Grundmann, President of the Society for the Cancer Campaign of Northrhine-Westfalia, West Germany, an International Conference on NPC Basic Research as applied to Diagnosis and Treatment was organized in Düsseldorf, West Germany in October 1980. Approximately 65 investigators representing all aspects of NPC from 15 countries participated. A summary of the highlights of the symposium were prepared and published in the May 1981 issue of Cancer Research. The proceedings of the symposium, edited by Prof. Grundman, Drs. Krueger and Ablashi are in press. Preparations are in progress for the next International Symposium on NPC, which is planned for 1982 in Kuala Lumpur, Malaysia.

Significance to Biomedical Research and the Program of the Institute:

The findings under A and B (1) on EBV, HVS, and HVA relate to clinical and laboratory studies which confirm and extend previous studies and initiate new approaches in biochemical, immunovirological, pathological and virus-chemical interactions designed to define the interactions and immunological parameters of the herpesviruses associated with neoplastic disease. It is hoped all of the information developed will help to provide valuable clinical tools for diagnosis, prognosis, and better control of herpesvirus-associated neoplasms. The cell-virus-chemical interactions support a multifactoral etiology of tumors implicating the herpesviruses etiologically. This work also suggests that host and other factors (tumor promoters, and weak carcinogens) do affect the outcome of herpesvirus infection, cell transformation and possible tumor induction. Some of the *in vitro* findings may also be applicable to *in vivo* interactions contributing to oncogenesis.

The monoclonal antibody to EBV and HVS glycoproteins would be a very useful tool in identification of strain differences. The transforming P_3HR-1 EBV would be useful in studying the strain differences between BL isolates of EBV and NPC isolates of EBV at biological and molecular levels. Moreover, the rescued P_3HR-1 EBV from P_3HR-1 transformed nonproducer cord cells containing a homogenous preparation of transforming virus particles will be useful for molecular, and biologic comparisons with virus derived from B95-8, and AG876 cell lines and putative recombinants between these viruses and P_3HR-1.

The international collaborative studies, conferences, etc. are needed to create a working relationship, better communication, exchange of materials, exchange of investigators, and above all opportunities to work with virus-associated human tumor, i.e., NPC, and BL which are more frequent in certain populations of the world.

Proposed Course:

A. The project will be continued. More emphasis will be placed on further in vitro and in vivo characterization of transforming EBV from P_3HR-1, rescued EBV from P_3HR-1 transformed cells. Secondly, it would be important to answer the question whether individual virus particles possess both properties or whether there is a mixture of virus particles. If the latter is the case, experiments as suggested by rescue experiments would be important to determine whether individual P_3HR-1 cells produce one or both kinds of virus and the nature of the difference between these particles.

Emphasis will be placed on further characterizing the monoclonal antibodies from HVS and EBV and their use in viral strain differentiations.

The interaction of tumor promoting agent and or mutating agents such as MNNG will be used in further study of EBV and HVS. Moreover, anti-inflammatory agents would be tested for their action on viral or TPA-induced antigens to determine their usefulness in treatment of NPC.

More emphasis will be placed on use of fibroblast interferon on NPC cells such as P_3HR-1/D98 hybrid cells which induce undifferentiated carcinomas similar to NPC in nude mice.

To study the effect of EBV antibodies (VCA-IgA, IgG-EA, and ADCC) in NPC patients to determine which antibodies increase with tumor burden, tumor recurrence, therapy, etc., in order to find their diagnostic and prognostic applications.

B. International participation in the NPC program will be continued, along with plans for the 1982 international symposium.

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Armstrong, G.R., Ablashi, D.V., Pearson, G.R., Krueger, G.R.F., Easton, J.M., Bouguermouh, A., Allal, L., Prasad, U., and Connelly, R.: Comparison of Epstein-Barr virus antigens in nasopharyngeal carcinoma from intermediate and low risk populations. In Grundmann, E., Krueger, G., and Ablashi, D. (Eds.): Cancer Campaign Series Vol. 5 on Nasopharyngeal Carcinoma: Basic Research as Applied to Diagnosis and Therapy. (In press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05110-02 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.R. Andersen	Senior Staff Fellow	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	E. Canaani	Expert	LCMB	NCI
	A. Varesio	Visiting Fellow	LCMB	NCI
	K. Robbins	Expert	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI
	S. Devare	Visiting Associate	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI,NIH Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this study is to biochemically characterize retroviruses in order to understand the mechanisms by which these viruses induce cancer in their natural hosts. The role of these viruses in the etiology of human cancers is also under study. Studies currently in progress are the following: 1) biochemical characterization of replication-defective mammalian transforming viruses; 2) search for the presence of retroviral genes and gene products in human tumors.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. To biochemically characterize mammalian transforming retroviruses.
2. To determine the mechanisms of oncogenesis by retroviruses in their natural hosts.
3. To determine whether or not retroviruses play a role in the etiology of cancers of higher primates, including humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of tumor viruses and their proteins; molecular hybridization techniques using single-strand specific nuclease and hydroxyapatite chromatography to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing, and electron microscopy.

Major Findings:

We have cloned and characterized the genome of the simian sarcoma-associated virus (SSAV) and the BALB murine sarcoma virus (BALB-MSV). The cloned genome of SSAV is currently being used in the characterization of the genome of the simian sarcoma virus.

BALB-MSV is a naturally occurring transforming retrovirus of mouse origin. The integrated form of the viral genome was cloned from DNA of a BALB-MSV transformed nonproducer NRK cell line in the Charon 9 strain of bacteriophage λ. In transfection assays, the 19 kbp recombinant DNA clone transformed NIH/3T3 mouse cells with an efficiency of 3×10^4 focus forming units per pmole. Such transformants possessed typical BALB-MSV morphology and released BALB-MSV following helper virus superinfection. A 6.8 kbp DNA segment within the 19 kbp DNA possessed restriction enzyme sites identical to those of the linear BALB-MSV genome. Long terminal repeats (LTRs) of approximately 0.6 kbp were localized at either end of viral genome by the presence of a repeated constellation of restriction sites and by hybridization of segments containing these sites with nick translated Moloney MuLV LTR DNA. A continuous segment of at least 0.6 and no more than 0.9 kbp of helper virus unrelated sequences was localized toward the 3' end of the viral genome with respect to viral RNA. A probe composed of these sequences detected 6 distinct Eco RI-generated DNA bands in normal mouse cell DNA as well as a smaller number of bands in rat and human DNAs. These studies demonstrate that BALB-MSV, like previously characterized avian and mammalian transforming retroviruses, arose by recombination of a type C helper virus with a well conserved cellular gene. We constructed in plasmids, a genomic subclone of the BALB-MSV which coded for the protein involved in the malignant transformation of mammalian cells. One of the subclones, utilized as a probe specific for the src gene of BALB-MSV, was used to detect related sequences in the Harvey and Kirsten sarcoma viruses.

Significance to Biomedical Research and the Program of the Institute:

The availability of highly sensitive and specific biochemical and immunological probes for retroviral gene products has become increasingly of value in order to both demonstrate the possible etiologic involvement of these viruses in human cancers and to study the role that retroviral gene products may play in normal cellular functions.

Proposed Course:

Current work is focused on the isolation of endogenous sequences in the mouse and human cellular genome which are related to the src gene of BALB-MSV. We are also determining the DNA sequences of the region of BALB-MSV which codes for malignant transformation. Definition of the DNA sequence will make it possible to elucidate the splicing mechanism and reading frame of the sequences which code for the protein responsible for malignant transformation. With this knowledge, it should be possible to obtain expression of an active transforming gene product in a suitable prokaryotic system. We will be able to utilize the purified active src protein to (1) establish radioimmunoassays for the detection of the src gene product and immunologically related proteins; (2) investigate the interaction of the src gene product with purified subcellular components to elucidate the possible series of events leading to transformation; and (3) determine the effectiveness of the prokaryotic synthesized src protein in immunizing mice against BALB-src derived tumors.

Publication:

Andersen, P. R., Tronick, S. R., and Aaronson, S. A.: Molecular cloning and structural organization of BALB/c mouse sarcoma virus (BALB-MSV). J. Virol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05112-02 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Murine Leukemia Viruses: Mechanism of Leukemogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Storch	Research Associate	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)

T.M. Chused, LMI, NIAID

LAB/BRANCH

Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to understand how murine leukemia viruses (MuLV) cause lymphocytic leukemia. We have sought to 1) identify preleukemic changes in the lymphoid cells of MuLV-infected animals using fluorescent labeled antibody to viral and lymphoid differentiation antigens and the fluorescence activated cell sorter, and 2) determine the region of the MuLV genome responsible for leukemogenesis of specific lymphoid cells by generating recombinants between a virus that causes T cell leukemia and a a virus that causes B cell leukemia.

PHS-6040
(Rev. 2-81)

Project Description

Objective:

To understand how murine leukemia viruses (MuLV) cause lymphocytic leukemia, we will identify 1) the subpopulations of lymphoid cells in MuLV-infected animals that govern susceptibility and resistance to T and B cell leukemia, and 2) the region of the MuLV genome responsible for transforming specific lymphoid cells.

Methods Employed:

When inoculated into newborn NFS/n or BALB/c mice, Moloney-MuLV virus causes a T cell leukemia and Rauscher-MuLV causes a B cell leukemia. To determine if cells of T lineage are more susceptible to Moloney-MuLV infection and cells of B lineage are more susceptible to Rauscher-MuLV, we analyzed cells of spleen, thymus and bone marrow from infected pre-leukemic mice for viral expression and T and B markers, simultaneously. We incubated cells with FITC-conjugated anti-viral serum and XRITC-conjugated antibody against lymphoid differentiation antigens. The amount of each fluorochrome that bound to individual cells was determined by flow microfluorometry and displayed on computer-generated contour plots.

We have also screened other inbred strains of mice, including mice with genetic defects in lymphocyte ontogeny and function, for resistance to Moloney- and Rauscher-induced leukemogenesis.

We have employed selective tissue culture conditions to generate a series of recombinants between a temperature-sensitive mutant of Rauscher-MuLV and a fragment of cloned Moloney-MuLV proviral DNA. We have determined the origin of the viral sequences of each recombinant by immunotyping its translation products.

Major Findings:

Thymocytes are significantly more susceptible to infection and alteration of surface Thy 1.2 by Moloney-MuLV. T and B cells in the spleen and bone marrow are equally susceptible to Moloney- and Rauscher-MuLV infection. Null cells in the spleen and large cells in the bone marrow are infected by Moloney- and Rauscher-MuLV and increase in frequency before the leukemic T and B cells appear.

CBA/N, CBA/CaHN and DBA/2 are susceptible to Moloney- and Rauscher-MuLV infection but resistant to leukemogenesis. Resistance appears to segregate as an autosomal dominant gene with incomplete penetrance and is not linked to H-2 or Fv-1 haplotype. In resistant strains infected with Moloney-MuLV, thymocytes are infected and altered in Thy 1.2 expression, but the proportion of null cells in the spleen does not increase.

A series of recombinants between Moloney- and Rauscher-MuLV has been isolated and cloned. Their gag region appears to have been derived from Moloney-MuLV and their env region from Rauscher-MuLV. The recombinants are leukemogenic when inoculated into newborn NFS/n mice.

Significance to Biomedical Research and to the Program of the Institute:

Understanding the mechanisms by which murine leukemia viruses transform specific lymphoid cell populations may provide important insights into the alterations that lead to naturally occurring malignancies of these cell types.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05135-02 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Murine and Human Monoclonal Antibodies Reactive with Mammary Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey Schlom	Section Chief	LCMB	NCI
Others:	David Colcher	Microbiologist	LCMB	NCI
	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
	Marianna Nuti	Fogarty Fellow	LCMB	NCI
	Daniela Stramignoni	Fogarty Fellow	LCMB	NCI

COOPERATING UNITS (if any) Dept. of Pathology, G. W. Univ., Wash., D.C.; Sidney Farber Cancer Center, Boston, Mass.; Dept. of Pathology, Univ. of Calif., Davis, CA; Dept. of Pathology, Univ. of Southern Calif., Los Angeles, CA

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Murine monoclonal antibodies have been generated which demonstrate reactivity with human mammary tumor cells and not with apparently normal tissues tested. The monoclonals could be placed into five major groups based on their differential binding to the surface of live mammary tumor cells in culture, extracts, or to tissue sections of mammary tumors employing the immunoperoxidase technique. Lymphocytes from lymph nodes obtained at mastectomy in breast cancer patients have been fused with murine non-Ig producer myeloma cells to obtain human-mouse hybridoma cultures that synthesize human monoclonal antibodies. One human monoclonal IgM was used in the immunoperoxidase technique to discriminate between normal and mammary carcinoma cells from 55 of 59 patients. Monoclonal antibodies have been generated against both the major internal protein and external glycoprotein of the mouse mammary tumor virus. These antibodies have been used in the murine model to define the heterogeneity of expression of given gene products that may occur among mammary tumors of a given species and, at times, in different areas of the same mammary tumor.

Project Description

Objectives and Historical Background: Numerous investigators have reported the existence of human mammary tumor associated antigens. These studies, all conducted with conventional hyperimmune polyclonal sera, however, were unfortunately hampered with regard to the heterogeneity of the antibody populations employed, and the amount of specific immunoglobulin that could be generated. Since the advent of hybridoma technology, monoclonal antibodies of predefined specificity and virtually unlimited quantity may now be generated against a variety of antigenic determinants present on normal and/or neoplastic cells. The rationale of these studies is to utilize extracts of human metastatic mammary tumor cells as immunogens in an attempt to generate and characterize monoclonal antibodies that are reactive with determinants that would be maintained on metastatic, as well as primary, human mammary carcinoma cells. The objective of a second group of studies is the generation of human monoclonal antibodies that are reactive with human mammary carcinoma cells. The rationale behind these studies is that axillary lymph nodes, draining breasts bearing a mammary tumor, may contain B-lymphocytes that are primed against antigens that may be shed from tumor cells. These human primed B-lymphocytes could then be fused with non-Ig secreting murine myeloma cells to form human mouse hybridomas that secrete human Ig. These hybridoma cultures could then be cloned in an attempt to obtain stable hybridoma cultures synthesizing human monoclonal antibodies. It is also the purpose of our studies to generate monoclonal antibodies to the major polypeptides of MTVs. These antibodies could first be used to further define type, group and interspecies reactivities among MTVs derived from strains of laboratory mice commonly used in mammary cancer research. These antibodies could also be used to define the diversity of expression of individual antigenic determinants in primary mammary tumors of various Mus strains and species and may also serve as a prototype for the detection of mammary tumor associated-antigens.

Major Findings:

I. Monoclonal Antibodies Reactive with Human Mammary Tumor Cells. Mice were immunized with membrane-enriched fractions of human metastatic mammary carcinoma cells from either of two involved livers and spleens of immunized mice were fused with NS-1 myeloma cells to generate hybridoma cultures. Supernatant fluids from these cultures were screened in solid phase RIAs for the presence of immunoglobulin reactive with extracts of metastatic mammary tumor cells from involved livers and not reactive with similar extracts of apparently normal liver. The monoclonal immunoglobulins from eleven hybridoma cell lines were chosen for further study. The isotypes of all eleven antibodies were determined; ten were IgG of various subclasses and one was an IgM. The primary screen for monoclonal antibodies reactive with human mammary carcinoma cells was a solid phase RIA employing cell extracts of two breast tumor metastases and apparently normal human liver as test antigens. The eleven monoclonal antibodies could immediately be divided into three major groups based on their differential reactivity. All eleven antibodies were negative when tested against similar extracts from normal human liver, a rhabdomyosarcoma cell line, a cell line derived from cultures of human milk, a mouse mammary tumor cell line, a mouse fibroblast cell line, a feline kidney cell line, and disrupted mouse mammary tumor virus and mouse leukemia virus.

To further define the reactivities of the eleven monoclonal antibodies, and to determine if they bind cell surface antigens, each antibody was tested for binding to live cells in culture. Test cells included three established cell lines of human mammary carcinoma, several cell lines of other human tumors, and eleven cell lines established from apparently normal human tissues. The nine monoclonals grouped together on the basis of their binding to both metastatic cell extracts could be separated into three different groups on the basis of their differential binding to the surface of live mammary cells in culture. None of the antibodies bound to several sarcoma and melanoma cell lines tested. Some of the antibodies appeared to possess a "pancarcinoma" pattern of binding activity. Two of the monoclonals, on the other hand, did not react with the surface of any of the mammary tumor cell lines tested, but could be distinguished from the other nine monoclonals by their differential binding to cell extracts. None of the eleven monoclonal antibodies bound to any of the following cell lines derived from apparently normal human tissues: breast, uterus, skin, embryonic skin and kidney, and fetal lung, testis, thymus, bone marrow, and spleen. Control monoclonals, however, did bind all of these cells. To further define the range of reactivity of each of the eleven monoclonal antibodies, the immunoperoxidase technique on tissue sections was employed. All the monoclonals reacted with mammary carcinoma cells of primary and metastatic mammary carcinomas. A high degree of selective reactivity was observed with mammary tumor cells, and not with apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes of the breast with all eleven monoclonal antibodies.

II. Generation and Characterization of Human-Mouse Hybridoma Cultures. To date, over 1,400 microtiter wells have been seeded with fusion products of murine NS-1 myeloma cells (non-Ig secreting) and lymphocytes from 16 patients. Of these, 301 human-mouse hybridoma cultures have been successfully propagated. All nodes were from patients with infiltrating duct or lobular adenocarcinoma. Hybridoma cultures were first tested for the synthesis of human IgG or IgM 14-28 days after fusion. Of the 301 replicating hybridoma cultures, 52 (17%) synthesized either human IgG or human IgM. The duration of human Ig synthesis ranged from [illegible] to at least 300 days. Twenty-three of the 52 cultures (44%) continued to synthesize human Ig through the 61 to 300 days of observation.

The level of human IgG or human IgM production was measured by liquid immunobead competition and solid-phase radioimmunoassays. The titers observed were comparable to those detected in our laboratory and by others with both mouse-mouse and human-human hybridomas. Many of the human-mouse hybridoma cultures were unstable for Ig production. For example, only 3 of 21 primary clones of hybridomas from one patient previously shown to be positive for Ig production on day [illegible], were positive when assayed on day 94. Upon recloning the three clones positive for Ig synthesis, however, [illegible] of 123 secondary clones (MBE6) remained positive for human Ig synthesis through the 300 day observation period.

Human monoclonal antibody MBE6 was first tested by immunoperoxidase for reactivity with tissue sections of the primary breast tumor mass (infiltrating duct carcinoma) of patient [illegible] and demonstrated marked cytoplasmic staining in most of the mammary tumor cells. Differences in both intensity of staining and percentage of cells stained were observed in various areas of the primary tumor mass. Antibody MBE6 could distinguish clearly between malignant mammary cells and "non-malignant" mammary epithelium or stromal cells. Antibody MBE6 was then tested for its ability to detect metastatic mammary carcinoma cells in the lymph nodes of patient MB and could clearly distinguish between metastatic breast cells and lymphocytes. A variation in intensity of staining was also observed within a given population of metastatic cells of a given lymph node. This same monoclonal antibody was also tested for its ability to bind to mammary carcinoma cells of patients other than MB. To date, monoclonal MBE6 has demonstrated reactions with mammary carcinoma cells of 55 of 59 patients tested.

III. Monoclonal Antibodies to Murine Mammary Tumor Antigens. Mice and rats were immunized with disrupted MTVs from M. musculus (MMTV[C3H]), M. cervicolor (MC-MTV) and M. cookii (MCo-MTV). Standard hybridoma technology was employed to obtain monoclonal antibodies that were reactive with test immunogens. The various monoclonal antibodies then were tested for their ability to bind to various MMTVs from several strains of mice. The viruses were also propagated in feline cells to ensure that the reactivities observed were not directed against murine determinants. One monoclonal antibody bound to the highly oncogenic MMTVs from C3H and GR mice but not to the highly oncogenic MMTVs from RIII and A mice. This monoclonal was also able to distinguish between two MMTVs with moderate oncogenicity, i.e., it bound to the MMTV purified from BALB/c mice but not MMTV from C3HfC57BL mice. This monoclonal was also able to distinguish between the horizontally transmitted MMTV(C3H) and the vertically transmitted MMTV(C3Hf) of C3H mice. MMTVs from six different strains of M. musculus could clearly be distinguished from each other by this method.

The immunoperoxidase technique was employed to determine if the monoclonal antibodies generated could be used to detect MTV antigenic determinants on tissue sections of primary and transplanted murine mammary tumors. Two major types of staining patterns were observed: apical staining, in which the immune reaction was concentrated at the periphery of lumens of acini, and focal staining, in which the immune reaction was detected as discrete intracytoplasmic foci. Two phenomena became apparent using these antibodies to monitor expression of a distinct antigenic determinant in tumor cells. The first was that a given antigenic determinant can be expressed in a different manner in two different mammary tumors. The second phenomenon was the quantitative heterogeneity in staining within a given mammary tumor, i.e., most mammary tumors tested from a variety of Mus strains and species presented some areas that were positive, and some that were negative, for expression of a given determinant.

Significance to Biomedical Research and Proposed Course: Monoclonal antibodies that are reactive with human mammary carcinoma cells may eventually be useful in the diagnosis, prognosis, and treatment of human breast cancer. Antibodies could be radioactively labeled for in situ scanning to detect metastatic lesions in nodes of the internal mammary chain and at distal sites. Preliminary experiments will first be carried out employing a nude mouse model and transplanted human mammary tumors. Experiments are also under way to determine if the presence or absence of any one or combination of the antigenic determinants recognized by these monoclonal antibodies on tissue sections have any prognostic value, i.e. does it reflect the degree of differentiation or malignancy of a given mammary tumor cell population. These monoclonals may also serve a useful purpose in providing a marker for the transformed state in in-vitro carcinogenesis experimentation. If monoclonal antibodies are ever used clinically, human monoclonal antibodies may have the advantage over murine monoclonal antibodies in that one would anticipate a reduced immune response to human immunoglobulin. The human monoclonal antibodies thus far generated and characterized have been of the IgM isotype. Further studies are underway to determine if useful human IgG monoclonal antibodies can be generated and characterized.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05148-02 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Study of Neoplasias of Outbred Colonies of Feral Species of the Genus Mus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Callahan	Microbiologist	LCMB	NCI
Others:	Jeffrey Schlom	Section Chief	LCMB	NCI
	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
	William Drohan	Sr. Staff Fellow	LCMB	NCI
	Bernard Sass	Vet. Medical Officer	OD	NCI
	Michael Potter	Section Chief	LCBGY	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have identified an outbred colony of Mus cervicolor popaeus established from a feral population in Asia which has a high incidence of mammary tumors. Other colonies of M. cervicolor popaeus derived from mice caught in other locations or other species of Mus from the same general location have a low or no incidence of mammary tumors. Most of the mammary tumors histologically fall into adenocarcinomas type A, B or C; however, novel types of tumors are also observed including ductal carcinomas. In many but not all of the tumors type B retroviral particles were observed. Interspecies radioimmunoassays for MTV-associated proteins showed that the tumor-associated virus is antigenically indistinguishable from the M. cervicolor milk-borne type B retrovirus MC-MTV which has previously been shown by us to be distantly related to the laboratory strains of MMTV from M. musculus.

PHS-6040
(Rev. 2-81)

Project Description

Background and Rationale: Several independent outbred colonies of feral mice from Thailand were developed in 1976-77 to study the genetic variation of endogenous retroviruses in natural populations and their potential involvement in neoplasia. For this purpose mice of a given species, trapped in different provinces of Thailand, were each developed into independent breeding colonies. These colonies included M. cervicolor cervicolor (Chantaburi province), M. cervicolor popaeus (Tak, Chantaburi and Saraburi provinces), M. cookii (Loei province), M. caroli (Chantaburi province) and M. pahari (Tak province).

Objectives: To develop outbred colonies of various species of Mus for the identification and study of new endogenous retroviral genes and genetic elements involved in the etiology of neoplasia.

Major Findings: We have recently completed a retrospective study of the incidences of neoplasias in the various colonies of mice. With one exception each colony had a tumor incidence of 1 to 2% in populations ranging from 300 to 1000 mice. In contrast, the M. cervicolor popaeus Tak colony has a 10% incidence of neoplasias in a population of 1300 mice. Significantly over half of these tumors were of the mammary gland. By tracing the breeding history of each of the affected females it became clear that they all were descendants of one of three females (trapped in Thailand) which were used to initiate this colony. Attempts to link the development of mammary tumors to paternal lineage have been negative.

A comparison of the tumor incidence in breeders versus virgins revealed that in the former group 42/167 got tumors while only 7/181 in the latter group were affected. The average age at which the tumors appear is 15 months and is also the average age at death for female breeders or virgins. The tumors could be placed in one of three groups depending on the timing of their appearance: (1) tumors which appeared in retired breeders, (2) tumors which appear during a pregnancy and continue to develop after parturition (3) tumors which appear during a pregnancy, but regress after parturition then reappear in subsequent pregnancies and continue to develop. The latter two groups of tumors first appeared at an average age of 12.5 months.

By histological criteria over half of the tumors were type B adenocarcinomas. In addition to these tumors were others which appeared to be mixtures of type A and B, or B and C adenocarcinomas. Other mammary tumors appeared novel in that they resembled the type Y adenocarcinoma or contained duct-like structures and still others had an organoid appearance. None of these novel tumors appeared predominently in any one lineage group of mice.

Protein extracts of the mammary tumors were tested for the expression of endogenous retroviruses (type C-I, type C-II, M432, and MC-MTV) in competition radioimmunoassays. Significantly, 18/21 tumors contained detectable levels of MC-MTV-associated proteins. Normal tissues from the same mice were negative in this assay. Similar conclusions were reached with immunoperoxidase assays using antisera prepared against MC-MTV. Type C-1 associated proteins were present in 5/21 tumors while type C-II and M432 related proteins were not detected. In addition, preliminary results of restriction enzyme analysis of cellular DNA from some of the mammary tumors suggests that the MC-MTV genome may be amplified in a manner similar to that observed in M. musculus C3H mammary tumors. These results suggest that MC-MTV may play an etiological role in some M. cervicolor popaeus mammary tumors.

Significance to Biomedical Research and Proposed Course: The M. cervicolor popaeus Tak colony offers a unique model system to experimentally identify the various etiological agents and factors which are involved in mammary neoplasias. Several characteristics set this colony apart from the various high incidence strains of M. musculus: 1) the large variety of histologically defined tumor types, 2) the low incidence of mammary tumors in virgins, 3) the variety of physiological states of the mouse during which the tumors first appear. Since all of the mice which developed tumors could be traced through their maternal lineage to one of three females which were trapped in the Tak province of Thailand, we conclude that the apparent complexity of genetic and physiological factors involved in the disease more closely reflects the situation in a natural population.

Although it is tempting to conclude that a principal etiological agent involved in the mammary neoplasias of the M. cervicolor popaeus Tak colony is the milk-borne MC-MTV, other colonies of this species of mice express a similar virus in their milk, but have either a low or no incidence of disease. Currently we are testing the biological activity of the M. cervicolor popaeus Tak milk in low incidence colonies of M. cervicolor popaeus and inbred strains of M. musculus. In other experiments the high incidence popaeus Tak mice are being nursed on low incidence popeaus mice. In addition, we are examining different histological types of mammary tumors by immunoperoxidase staining using antisera prepared against MC-MTV to determine whether there are specific cell types which are expressing the virus. In other experiments, genetic organization of the MC-MTV genome in tumors and lactating mammary glands are being examined by restriction endonuclease analysis to determine whether specific types of tumors correlate with a pattern of restriction DNA fragments.

Publications:

Horan Hand, P., Teramoto, Y. A., Callahan, R., and Schlom, J.: Interspecies radioimmunoassays for the major internal protein of mammary tumor viruses. Virology 101: 61-71, 1980.

Teramoto, Y. A., Horan Hand, P., Callahan, R., and Schlom, J.: Detection of novel murine mammary tumor viruses by interspecies immunoassays. J. Natl. Cancer Inst. 64: 967-975, 1980.

Callahan, R., Hogg, E., Sass, B., Teramoto, Y. A., Todaro, G. J., and Schlom, J.: A feral population of M. cervicolor popaeus with a high incidence of spontaneous mammary tumors. J. Natl. Cancer Inst. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05159-01 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Production of Monoclonal Antibodies Against Human Sarcoma and Leukemia Related Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Balachandran	Visiting Fellow	LCMB	NCI
Other:	E. Premkumar Reddy	Visiting Scientist	LCMB	NCI
	S.A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Spleen cells from mice immunized with human rhabdomyosarcoma cells were fused with X63 cells. The hybridomas secreted antibodies which specifically interacted with human sarcoma cell extracts but not with extracts from normal cells. These antibodies also cross-reacted with cell extracts from other human tumor cells. These findings show that antigenic similarities exist between different tumor cells of human origin. Currently studies are being conducted to identify these tumor-specific antigens.

Project Description

PROJECT 1

Objective:

To explain the role of cellular antigens on the initiation and maintenance of transformation.

Methods Employed:

Single cell suspensions from spleens of mice immunized with human sarcoma cells were used for making hybridomas. Antibody secreting hybridomas were grown either in culture or in the intra-peritoneal cavity of pristane primed mice. Culture medium or ascites fluid were used to define antigenic similarities among different tumor cells.

Major Findings:

The cell hybridization technique was used to generate hybridoma cell lines which synthesize antibodies directed against human tumor antigens. For this purpose, mice were immunized with human rhabdomyosarcoma cells and their spleen cells fused with mouse myeloma cell line P3x63. Stable hybridoma cell lines that propagated *in vitro* were assayed for antibodies that reacted specifically against human rhabdomyosarcoma cells but not normal human fibroblasts. Nine such cell lines which secreted antibodies specific to human rhadomyosarcoma cells were selected and propagated *in vitro* and *in vivo*. The antibodies produced by these clones were assayed against other human tumor cell lines such as malignant melanoma, astrocytoma and Burkitt's lymphoma. The results indicate that their antibodies cross-react with most of the tested human tumor cell lines, indicating antigenic similarities between various human tumors.

Significance to Biomedical Research and the Program of the Institute:

Characterization of tumor antigens should allow us to elucidate their role in cellular transformation.

Proposed Course:

Experiments will be designed to characterize the origin, nature and possible function of these tumor antigens.

PROJECT 2

Studies on the lymphoid transformation by mouse leukemia viruses.

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Objective:

To study the nature of B and T cell transformation by mouse leukemia viruses.

Methods Employed:

A tissue culture method was developed to grow the transformed cells in continuous culture. Transformed cells that multiplied in culture were characterized as B and T cells by standard procedures. The stage of development of the lymphomas was assessed by radioimmunoassay and biosynthetic studies.

Major Findings:

1. Rauscher virus transforms only cells of B cell lineage in all strains of mice tested.

2. With a few exceptions, T cells are the targets of Moloney virus induced transformation in different strains of mice.

3. Amphotropes and ecotropes transform only B cells.

Significance to Biomedical Research and the Program of the Institute:

This study permits us to elucidate the *in vivo* location of viral targets in the host.

Proposed Course:

This project is now nearing completion.

Publications:

None.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05160-01 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunohistochemical Localization of Tumor Antigens with Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marianna Nuti	Fogarty Fellow	LCMB	NCI
Others:	Jeffrey Schlom	Section Chief	LCMB	NCI
	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
	David Colcher	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)
Dept. of Pathology, George Washington University, Washington, D. C.

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our laboratory has been involved in the generation of monoclonal antibodies that are reactive with human mammary tumor cells. Screening of monoclonal antibodies involves determining reactivity with live and fixed mammary cell lines in culture as well as with extracts of mammary tumor tissues. We have further characterized several different monoclonal antibodies, using the immunoperoxidase technique, on the basis of their reactivity to tissue sections of primary and metastatic breast lesions and their lack of reactivity of normal mammary and non-mammary tissues of the same individual. We have also been able to characterize the different monoclonal antibodies on the basis of which mammary tumors are reactive, and on their pattern of staining. This technique has also enabled us to further define the antigenic heterogeneity that exists among different mammary tumors and within a mammary tumor of a given individual.

PHS-6040
(Rev. 2-81)

Project Description

Objectives and Historical Background: Numerous investigators have reported the existence of human mammary tumor associated antigens. These studies, all conducted with conventional hyperimmune polyclonal sera, however, were unfortunately hampered with regard to the heterogeneity of the antibody populations employed, and the amount of specific immunoglobulin that could be generated. Since the advent of hybridoma technology, monoclonal antibodies of predefined specificity and virtually unlimited quantity may now be generated against a variety of antigenic determinants present on normal and/or neoplastic cells. The rationale of our laboratory has been to utilize extracts of human metastatic mammary tumor cells as immunogens in an attempt to generate and characterize monoclonal antibodies reactive with determinants that would be maintained on metastatic, as well as primary, human mammary carcinoma cells. Multiple assays using tumor cell extracts and live and fixed cells in culture have been employed to reveal the diversity of the monoclonal antibodies generated. It is the purpose of these studies to use the immunoperoxidase method for in situ localization of antigens associated with mammary carcinoma.

Major Findings: Mice were immunized with membrane-enriched fractions of human metastatic mammary carcinoma cells from either of two involved livers. Spleens of immunized mice were fused with NS-1 myeloma cells to generate primary hybridoma cultures. Supernatant fluids from these cultures were screened in solid phase RIAs for the presence of immunoglobulin reactive with extracts of metastatic mammary tumor cells from involved livers and not reactive with similar extracts of apparently normal liver. Whereas many cultures demonstrated immunoglobulin reactive with all test antigens, 370 cultures contained immunoglobulin reactive only with the metastatic carcinoma cell extracts. Following passage and double cloning of these cultures by endpoint dilution, the monoclonal immunoglobulins from eleven hybridoma cell lines were chosen for further study. The isotypes of all eleven antibodies were determined; ten were IgG of various subclasses and one was an IgM.

The primary screen for monoclonal antibodies reactive with human mammary carcinoma cells was a solid phase RIA employing cell extracts of two breast tumor metastases and apparently normal human liver as test antigens. The eleven monoclonal antibodies could immediately be divided into three major groups based on their differential reactivity to Met 1 vs. Met 2. All eleven antibodies were negative when tested against similar extracts from normal human tissues. To further define the reactivities of the eleven monoclonal antibodies, and to determine if they bind cell surface antigens, each antibody was tested for binding to live cells in culture. Test cells included three established cell lines of human mammary carcinoma (BT-20, MCF-7, and ZR-75-1). The nine monoclonals grouped together on the basis of their binding to both metastatic cell extracts could now be separated into three different groups on the basis of their differential binding to the surface of live cells in culture. None of the eleven monoclonal antibodies bound to any of the following cell lines derived from apparently normal human tissues: breast, uterus, skin, embryonic skin and kidney, and fetal lung, testis, thymus, bone marrow, and spleen. To further define specificity and range of reactivity of each of the eleven monoclonal antibodies, the immunoperoxidase technique on

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tissue sections was employed. All the monoclonals reacted with mammary carcinoma cells of primary mammary carcinomas (both infiltrating ductal and lobular). The percentage of primary mammary tumors that were reactive varied for the different monoclonals, ranging from 74% (23/31) and 80% (8/10) using monoclonals B6.2 and B38.1, respectively, to 22% (2/9) for monoclonal B50.4. In many of the positive primary and metastatic mammary carcinomas, not all tumor cells stained. In certain tumor masses, furthermore, heterogeneity of tumor cell staining was observed in different areas of a tumor, and even within a given area. A high degree of selective reactivity with mammary tumor cells, and not with apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes of the breast was observed with all eleven monoclonal antibodies. A dark reddish-brown stain (the result of the immunoperoxidase reaction with the diaminobenzidine substrate) was observed only on mammary carcinoma cells, whereas only the light blue hematoxylin counterstain was observed on adjacent normal mammary epithelium, stroma, and lymphocytes. Occasionally, a few of the apparently normal mammary epithelial cells immediately adjacent to the mammary tumor did stain weakly with the same pattern of staining seen in the tumor cells. The polymorphonuclear leukocytes and histiocytes in the stroma in the area of mammary tumor showed positive cytoplasmic staining. This would suggest that the reactivity is due to antigen shed by the tumor and phagocytized by reactive cells in the immediate proximity. The staining patterns of mammary carcinoma cells varied among the different monoclonals. Monoclonal B50.4 was reactive with mammary carcinoma cells displaying a dense focal staining. Monoclonal B6.2, together with B39.1, B84.1, B14.2, B25.2, on the other hand,reacts with alternate sections of the exact mammary carcinoma displaying a more diffuse cytoplasmic pattern. B38.1 instead showed predominently a dense (peri) membrane staining and B72.3 a membrane as well as cytoplasmic stain. These differential patterns of staining were consistent in the several primary mammary tumors and metastic lesions tested. The monoclonal antibodies could also be distinguished from one another on the basis of which mammary tumors they reacted with. For example, monoclonals B72.3 and B6.2 both reacted with infiltrating ductal mammary carcinoma 10970, but only monoclonal B6.2 reacted with mammary tumor 2657, while conversely, only monoclonal B72.3 reacted with mammary tumor 9388. To ensure against artifacts or differences due to sampling of tissues, these experiments were carried out by reacting monoclonals with alternate tissue sections. Frozen sections of primary mammary tumors were also tested with some of the monoclonals; as expected from the surface binding experiments to live cells in culture, the frozen sections revealed membrane staining.

Experiments were then carried out to determine if the eleven monoclonals could detect mammary carcinoma cell populations at distal sites, i.e., in metastases. Since the monoclonals were all generated using metastatic mammary carcinoma cells as antigen, it was not unexpected that the monoclonals all reacted, but with different degrees, to various metastases. Perhaps the most efficient of the monoclonals for this purpose was B6.2, which reacted with metastatic mammary carcinoma cells in lymph nodes of 6 of 7 patients, but did not react with uninvolved nodes of 8 patients. The immunoperoxidase staining technique also clearly revealed small clusters of a few mammary tumor cells that would have been extremely difficult to detect using conventional staining methodology. None of the monoclonals reacted with normal lymphocytes or stroma from any involved or uninvolved nodes. The eleven monoclonals also varied in their ability to detect metastatic mammary carcinoma lesions in distal sites such as liver, uterus, and bone. The monoclonals were all tested with normal and neoplastic non mammary tissues. No reactivity was observed with normal tissues of the following organs: spleen, bone marrow, thyroid, intestine, lung, liver, bladder, tonsils, stomach, and prostate. Some of the monoclonals showed reactivity with selected non-breast carcinomas such as adenocarcinomas of the colon. Other neoplasms tested and which showed no staining were sarcomas, lymphomas, glioblastomas, and melanomas.

Significance to Biomedical Research and Proposed Course: Three points should be made concerning the immunoperoxidase studies described here: (a) different monoclonals react with different staining patterns of alternate tissue sections of the same tumor; (b) monoclonals differed in the range of mammary tumors that showed reactivity, and (c) a heterogeneity of staining of tumor cells was observed in many tumor masses. This heterogeneity has also been observed in staining of primary and metastatic murine mammary tumors, and in human mammary tumor tissues stained with human monoclonal antibody. Because the live cell assays have demonstrated that at least nine of the monoclonals described here bind to the surface of human carcinoma cells preferentially, their potential clinical application for the immunodetection of primary and metastatic carcinoma lesions certainly merits consideration. It will also be of interest to determine if the presence or absence of any one or combination of the antigenic determinants recognized by the monoclonal antibodies described, on primary mammary tumor lesions or on involved nodes removed at mastectomy, are of any prognostic significance, i.e., do they reflect the degree of differentiation or malignancy of a given carcinoma cell population.

Publications:

Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J.: A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc. Natl. Acad. Sci. (USA) (In Press).

Nuti, M., Colcher, D., Horan Hand, P., Austin, F., and Schlom, J.: Generation and characterization of monoclonal antibodies reactive with human primary and metastatic mammary tumor cells. In: Proceedings of the Conference on Radioimmunoassay. North Holland, Elsevier (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05162-01 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Oncogenic Herpesviruses of Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Faggioni	Visiting Fellow	LCMB	NCI
OTHERS:	D.V. Ablashi	Microbiologist	LCMB	NCI
	P.H. Levine	Head, Clinical Studies Section	LVC	NCI
	K.S. Sundar	Visiting Fellow	LVC	NCI
	J. Rice	Head, Perinatal Carcinogenesis Section	LEP	NCI

COOPERATING UNITS (if any)
W.F. Loeb and G. Parker, Litton Bionetics, Inc., Kensington, MD; D. Viza, Faculte de Medicine Broussais, Paris, France; G. Pizza, Ospedale M. Malfrighi, Bologna, Italy

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular & Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) Four different strains of New England white inbred rabbits, the Ax, III/J, III/B and Y strains, were used to study their susceptibility to HVS and EBV, with and without tumor promoting agent (TPA). All the HVS inoculated rabbits developed malignant lymphoma. Both undifferentiated and well-differentiated lymphoma were found which varied with the strain of the rabbits, and the presence or absence of TPA potentiated the initiation of the lymphoma and the degree of lesions. The EBV-inoculated rabbits thus far failed to develop lymphoma, but VCA and EA antibodies were detectable and in some of the rabbits Downey-like cells were observed. (2) Specific transfer factor against Herpesvirus saimiri has been replicated in vitro, inducing the human lymphoblastoid cell line LDV/7. This transfer factor may be useful in prevention or therapy of T cell lymphomas.

Project Description

Objectives:

1. To examine an animal system less expensive and more easily obtainable for clinical, immunological and pathogenetic studies of Herpesvirus saimiri-induced tumors. Also to look for other tumor markers in the animals which may be later used for initiation of therapy.

2. To evaluate if tumor promoting agents or chemical carcinogens can induce or potentiate the action of HVS and EBV.

3. To follow up the humoral and cellular immune responses in all the inoculated animals, and the possible biochemical detection of the appearance of a neoplastic disease with oncofetal proteins, enzymes and prostaglandins.

4. Use of specific transfer factor against HVS for prevention or therapy of HVS-inoculated animals.

Methods Employed:

Several New Zealand white rabbits, fully or partially inbred strains III/J, Ax, III/B and Y, have been injected with prototype partially purified Herpesvirus saimiri and with transforming strains of Epstein-Barr virus (B-95-8 and Ag 876). Some of the rabbits also received various doses of 12-O-tetradecanoylphorbol-13-acetate (TPA), a croton oil derivative, of n-methyl-n'-nitro-n-nitrosoguanidine (MNNG) and of 1-ethyl-1-nitrosourea (ENU). Dialysable transfer factor (TFD) against HVS was extracted from $5x10^6$ mononucleated cells obtained from the peripheral blood of two immunized rhesus monkeys. Various human and nonhuman primate cell lines containing virus and/or viral antigens were used for virus preparation, antibody assays and virus isolation. Cell cultures were also attempted from tumors of the rabbits.

Major Findings:

1. All the Ax strain rabbits had moderately to well-differentiated lymphoma in comparison to the III/J strain where it was diagnosed as poorly or undifferentiated type.

2. The use of TPA after HVS inoculation resulted in enhancement of the tumor by reducing the incubation period significantly. The lesions were recognizable macroscopically.

3. Combination of TPA and HVS in a III/J rabbit induced a well differentiated lymphoma and a lymphoblastoid cell line was established from this animal's spleen.

4. The EBV-inoculated rabbits with and without TPA or ENU thus far failed to develop any disease, however, in some animals cells appearing like Downey cells were observed. These rabbits also developed antibodies to VCA and

EA, however EA antibodies either decreased in titer considerably after a certain period or completely disappeared. Such animals also were found to develop a low degree fever. These findings suggest perhaps a lymphoproliferative disorder, similar to infectious mononucleosis in humans.

5. Specific transfer factor against *Herpesvirus saimiri* has been replicated *in vitro*, inducing the LDV/7 cell line, and its *in vivo* properties have been studied in two owl monkeys. The antibodies against EA and LA HVS appeared earlier and the titers were higher in the two animals inoculated with TF and HVS than in the control one, which received the virus alone. The two treated monkeys developed lymphoma and died, while the control is still alive. This may suggest that somehow the TF could have altered the immune system of the monkeys and potentiated the effect of the virus.

Significance to Biomedical Research and the Program of the Institute:

The rabbit animal system for HVS and EBV-induced diseases can be of great utility, especially because of the limited availability and the high cost of the nonhuman primate system. The rabbits can be used for the evaluation of immunochemotherapy and antiviral and antitumor drugs, including interferon and cytosine arabinoside, on T cell lymphomas.

The documentation of successful antiviral/antitumor activity of specific transfer factor against HVS could provide a model system for oncogenic herpesviruses and cancer prevention.

Proposed Course:

1. Further evaluation of clinical, immunological and pathogenetic data of HVS-induced lymphomas in rabbits.

2. Biologic and molecular characterization of the cell line established from the spleen of a rabbit with well-differentiated HVS lymphoma that now is at the tenth passage of *in vitro* culture.

3. To study in the rabbit system the possible use of specific transfer factors for the prevention and/or therapy of T cell lymphomas.

Publications:

1. Faggioni, A., Ablashi, D., Armstrong, G., Sundar, S.K., Merrill, R., Martin, D., Valerio, M., Parker, G.A., and Fox, R.R.: *Herpesvirus saimiri* induced malignant lymphoma of the poorly and well differentiated types in three inbred strains of New Zealand white rabbit. *Proceedings Xth Intl. Symp. for Comp. Res. on Leukemia and Related Diseases*, 1981. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05163-01 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Leukemogenesis by Rauscher and Moloney Murine Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Habara	Visiting Fellow	LCMB	NCI
OTHER:	P. Reddy	Visiting Scientist	LCMB	NCI
	S. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clonal strains of Moloney and Rauscher murine leukemia viruses (M- and R-MuLV) have been demonstrated to induce tumors of T and B lymphoid cells, respectively. To investigate the basis for their cell specificity, these viruses have been cloned and analyzed for their molecular organization and attempts have been made to construct recombinant viruses for virologic testing. The physical map of unintegrated M-MuLV cloned in its Hind III site was consistent with that previously published. R-MuLV was cloned in its integrated form containing cellular flanking sequences at both ends. The cloned molecule is approximately 12.5 ± 1.25 kbp in length and contains more than 9 kbp of viral DNA. The cloned DNA induced virus production in NIH/3T3 cells upon transfection. The p12 and gp70 proteins of virus released were shown to possess antigenic determinants specific for R-MuLV. Heteroduplex analysis showed complete homology between R-MuLV DNA and Rauscher 70S RNA or M-MuLV DNA. On the other hand, restriction maps showed a strong correlation of cloned R-MuLV DNA with unintegrated linear R-MuLV DNA but not with M-MuLV DNA.

Project Description

Objectives:

To study mechanisms of oncogenesis of Rauscher and Moloney murine leukemia viruses which induce tumors in B and T lymphoid cells, respectively.

Methods Employed:

Restriction enzyme digestions, RPC-5 column chromatography, sucrose density gradient, nucleic acid hybridization (Southern and Northern blotting), molecular cloning techniques, gene machine preparative gel electrophoresis, heteroduplex analysis, immunoprecipitation.

Major Findings:

1. R-MuLV was cloned in its integrated form as follows: cellular DNA was isolated from normal rat kidney (NRK) cells after infection with clonal R-MuLV and was digested with Eco RI which has already been shown not to cleave unintegrated viral genome. An Eco RI fragment containing Rauscher-specific DNA was enriched by RPC-5 column chromatography and sucrose density gradient and cloned in lambda bacteriophage Charon 4A and finally recloned in bacteria plasmid pBR322.

2. From the restriction map and heteroduplex analysis, it was estimated that the cloned DNA is 12.5 ± 1.25 kbp in length and consists of more than 9 kbp proviral DNA and adjacent host flanking sequences of approximately 23 kbp and 0.3 kbp at 5' and 3' ends, respectively.

3. The cloned DNA induced virus production in NIH/3T3 cells upon transfection.

4. Unintegrated linear R-MuLV DNA fraction was isolated by gene machine preparative gel electrophoresis from cellular supernatant of R-MuLV-inoculated NRK cells and a preliminary restriction map developed. The cloned DNA was shown to have the same restriction map as that of unintegrated linear R-MuLV DNA.

5. M-MuLV was also cloned at its Hind III site from unintegrated form. Physical map of this clone was consistent with that previously published.

6. Heteroduplex analysis of the cloned R-MuLV DNA with Rauscher 70S RNA or the cloned integrated form Moloney-MuLV DNA showed complete homology.

Significance to Biomedical Research and the Program of the Institute:

Rauscher and Moloney MuLV cause tumors after long lag time and lack discrete transforming genes. The cloned DNA molecules will be utilized to further study the mechanism of leukomogenesis by these two viruses.

Proposed Course:

DNA structures of Rauscher and Moloney MuLVs will be compared using their restriction maps. Their LTR regions which might play an important role in oncogenesis will be analyzed by sequencing techniques. Recently, it has been reported that tumors induced by chronic avian leukosis virus synthesize new RNA species consisting of viral sequences covalently linked to cellular sequences. Studies will be carried out to see if Rauscher and Moloney murine leukemia viruses induce transformation by a similar mechanism.

Recombinant viruses between Rauscher and Moloney MuLVs will be constructed under the DNA clones described above. The susceptibility of newborn mice to tumor induction by the recombinant viruses will be studied. The lymphoid tumors induced by the recombinant viruses will be typed for their cell surface markers. These studies are expected to provide further understanding of the molecular mechanisms that are involved in determining the tissue specificity of acute leukemia viruses.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05164-01 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Interactions of Murine Hematopoietic Cells and Mammalian Retroviruses *In Vitro*

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Pierce	Staff Fellow	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	P.E. Reddy	Visiting Scientist	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
	B. Balachandran	Visiting Fellow	LCMB	NCI
	K. Nagao	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are: 1) to analyze the hematopoietic cell populations early and late after Rauscher or Moloney murine leukemia virus infection in an in vitro soft agar system in order to detect quantitatively the stage at which malignant cells appear during the course of the disease; 2) to determine the influence of helper virus pseudotype or hematopoietic growth factors on the hematopoietic target cell specificity of Abelson murine leukemia virus in vitro; 3) to screen a number of defective mammalian retroviruses for their ability to transform murine hematopoietic cells in vitro.

Project Description

Objectives:

1. Determine the differences in the malignant stages of murine chronic leukemia virus transformation through the use of an in vitro colony method capable of detecting bone marrow or spleen cells malignantly transformed by these viruses.

2. Determine the extent of the hematopoietic target cell specificity of Abelson murine leukemia virus (Ab-MuLV).

3. Screen a variety of mammalian retroviruses for their ability to transform hematopoietic cells in vitro.

Major Findings:

1. Hematopoietic cells were derived from bone marrow or spleens of Rauscher or Moloney murine leukemia virus (MuLV)-infected mice 10 to 14 weeks post infection. All mice studies showed pathological signs of chronic lymphoma including extensive splenomegaly. Rauscher or Moloney MuLV-transformed hematopoietic cell colonies were isolated and quantitated in an in vitro soft agar colony assay. Continuous lines of transformed hematopoietic cells could be established in vitro from a significant number of these colonies. Studies to date indicate that leukemogenic transformation may occur only at a relatively late stage of chronic leukemia virus infection.

2. The target cell specificity and stage of differentiation of hematopoietic cells transformed in vitro by Abelson murine leukemia virus (Ab-MuLV) was investigated. Ab-MuLV transformed clonal lines were obtained from an in vitro soft agar colony assay. Radioimmunoassay studies of immunoglobulin synthesis and Fc receptor assays revealed that Ab-MuLV in vitro transformed hematopoietic cell clones represented different stages in the differentiation pathway of the B cell lineage. The influence of different helper virus pseudotypes on Ab-MuLV transformation revealed that despite variation in the target cell specificities of the chronic leukemia viruses used to form Abelson pseudotypes, these helper viruses did not shift the in vitro target cell specificity of Abelson MuLV from the B cell lineage.

3. In vitro transformation of mouse hematopoietic cells by defective mammalian retroviruses other than Abelson MuLV was investigated utilizing the in vitro soft agar colony method. Three defective sarcoma viruses were shown to cause hematopoietic colony formation in the soft agar assay. Colony formation by these viruses was less efficient and occurred after a longer latent period than Ab-MuLV. Transformed cells derived from these in vitro transformed colonies could be propagated in vitro but only in the presence of a feeder layer comprised of normal adherent bone marrow cells. Cells derived from the feeder layer cultures were able to grow efficiently in soft agar.

Proposed Course:

1. Combination of in vitro and in vivo studies in order to more fully determine the time course of the appearance of tumorigenic cells during MuLV infection.

2. Use of hematopoietic cell growth factors to determine if in vitro transformation of Ab-MuLV can be shifted to other hematopoietic cell lineages and to determine if mature antibody-secreting B cells can be transformed by Abelson-MuLV.

3. To determine if other mammalian retroviruses have hematopoietic cell transforming activity and to more fully characterize the transforming events caused by these new viruses which appear to possess hematopoietic transforming activity.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05165-01 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Study of Sequences in Human DNA Related to the Transforming Genes of Murine Transforming Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Prakash	Visiting Fellow	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	E.P. Reddy	Visiting Scientist	LCMB	NCI
	S. Devare	Visiting Associate	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

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Molecular Biology Section

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NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The use of cloned DNA probes specific for the transforming regions of Moloney-MSV and Abelson-MuLV enabled us to detect homologous sequences in human DNA. These viral transforming gene-related sequences were cloned from human DNAs either by standard gene enrichment techniques or by screening cloned human DNA libraries. We have made a structural comparison of M-MSV and normal mouse cellular src and sarc sequences respectively with its human homologue by using restriction enzyme mapping and heteroduplex techniques.

Project Description

Objective:

To analyze the sequences in human DNAs related to the transforming genes of murine transforming viruses.

Methods Employed:

Standard gene enrichment techniques like RPC-5 chromatography and preparative gel electrophoresis were utilized. The rector Charon 16A was used for cloning the M-MSV src-related sequence in human DNA. Human DNA libraries were also screened for the detection of human homologues of the A-MuLV src region. The fragment or clone of interest was selected by plaque hybridization with the specific nick-translated probes. Characterization of the M-MSV-related human homologue was made by restriction enzyme mapping and heteroduplex analysis.

Major Findings:

1. Eco RI cut human placenta DNA has a 2.5 kbp fragment related to the M-MSV transforming gene.
2. By heteroduplex analysis, this 2.5 kbp fragment has been shown to have a homology to an extent of about 600 base pairs with the M-MSV transforming region.
3. Restriction enzyme mapping shows that the sites of cleavage are not similar for the human homologue (of M-MSV) and the M-MSV src and cellular sarc.
4. Detection of 4 kbp and 15 kbp fragments by screening human DNA libraries with A-MuLV src probe.

Significance to Biomedical Research and the Program of the Institute:

Detailed characterization of the human homologues should allow us to determine the specific regions of human DNAs related to the transforming genes of murine transforming viruses and their possible involvement in cancer.

Proposed Course:

Heteroduplex analysis and restriction enzyme mapping of the A-MuLV src-related human homologues will be performed. In addition, the biological activities of the cloned human DNA fragments will be tested.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05166-01 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation of Oncogenic Cellular Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Pulciani	Visiting Fellow	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	K.C. Robbins	Expert	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI

COOPERATING UNITS (if any)

None

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SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have isolated DNA from transformed cells and transfected it into normal mouse cells. Using this technique, we were able to transmit the malignant phenotype. Our goal is the isolation and characterization of cellular oncogenic gene (or genes) responsible for malignant transformation.

PHS-6040
(Rev. 2-81)

Project Description

Objective:

To isolate cellular oncogenes by molecular cloning techniques.

Methods Employed:

By DNA transfection techniques, it is possible to integrate purified DNA into living cells. We used this technique for studying the transforming activity of DNA isolated from a variety of malignant transformed cells. We transfected the purified DNA into normal cells and we were able to transform them to malignant phenotypes. Our purpose is to isolate and to characterize the cellular oncogenic gene or genes responsible for the malignant transformation.

For isolating any gene by molecular cloning techniques, it is necessary to know its structure in order to screen it by hybridization techniques. We don't know the structure of the oncogenic cellular gene, but it is possible to give it a structure by ligating it to some DNA of known structure and use that as marker DNA. Cellular DNA is first digested with restriction endonuclease enzyme which does not destroy the transformant activity and then ligated to similarly digested marker DNA (plasmid pBR322). This concatenated DNA is used to transform normal mouse cells. Some transformants can be expected to have the mouse oncogenic gene linked to marker sequences. The pBR322 sequences can be used to clone the animal host sequences containing the oncogenic cellular genes.

Major Findings:

Restriction endonuclease-cleaved DNA was tested for the ability to transfer the malignant phenotype to normal recipients in order to identify the enzymes that don't cleave the cellular oncogenic gene: Bam HI, Kpn I and Sst I don't cut into the gene. We ligated Bam HI cleaved cellular DNA to Bam HI cleaved pBR322 and then we transfected it into normal cells. The transformants that contained pBR322 sequences were selected by blot analysis. We performed a second round of transformation and again selected all transformants for the presence of pBR322 sequences. This second round of transformation is necessary to establish the relationship between pBR322 and the cellular oncogenic gene and also to avoid all transformants where pBR322 sequences have co-transfected with unlinked DNA fragments. Five primary transformants containing pBR322 sequences were used for the second cycle of transformation and several transformants, that still contain pBR322 sequences, were selected.

Significance to Biomedical Research and the Program of the Institute:

The cloning of cellular oncogenic genes will enable us to better understand the mechanisms involved in malignant transformation.

Proposed Course:

To clone oncogenic cellular genes by using the pBR322 sequences residing in the transformants to "rescue" the cellular sequence containing the cellular gene by standard molecular cloning techniques.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05167-01 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of the Transforming Gene of Simian Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.C. Robbins	Expert	LCMB	NCI
Other:	S.A. Aaronson	Chief	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	E. Canaani	Expert	LCMB	NCI
	A. Varesio	Visiting Fellow	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI
	S. Devare	Visiting Associate	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have obtained a molecular clone of integrated simian sarcoma virus (SSV) which possesses high-titered focus-forming activity. Analysis of our DNA clone revealed that SSV contains a nucleotide sequence, designated sis, which was derived from the genome of a New World primate. There is significant homology between sis and a uniquely represented nucleotide sequence found within the normal human genome. We have molecularly cloned the human analogue to sis and have begun to analyze its molecular and biologic properties. Efforts to search for involvement of this transforming retrovirus gene and its human analogue in naturally occurring human tumors are in progress.

Project Description

Objectives:

1. To biochemically and biologically characterize the transforming gene of SSV.

2. To analyze the human gene which is highly related to the cell-derived sequence of SSV for its involvement in human malignancy.

3. To determine the mechanism of SSV-induced oncogenesis and to apply this knowledge to understanding the etiology of cancers in humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of tumor virus gene products; *in vitro* synthesis and immunoprecipitation analysis of retrovirus gene products; molecular hybridization techniques using single-strand specific nuclease to detect and characterize viral genes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of the structure and biologic activity of cloned DNAs using transfection, restriction endonuclease mapping, nucleotide sequencing, and electron microscopy techniques.

Major Findings:

Simian sarcoma virus (SSV) is a replication-defective retrovirus which causes transformation in tissue culture and malignancies *in vivo*. This virus has been difficult to characterize because SSV stocks contain a high excess of an associated helper virus, simian sarcoma associated virus (SSAV). In order to analyze the structural and biologic properties of the genome of SSV, we molecularly cloned its integrated form from cells nonproductively transformed by the virus. By transfection analysis, recombinant viral DNAs demonstrated the ability to transform cells in tissue culture at high efficiency. Such transformants possessed a phenotype which was indistinguishable from that of transformants induced by the parental virus. A physical map of the viral DNA clone, deduced from restriction endonuclease analysis, revealed a 5.1 kilobase pair (kbp) SSV genome containing 0.55 kbp long terminal repeats (LTRs) flanked by 0.45 and 0.25 kbp of contiguous host cell sequences. By R-loop analysis, the viral DNA molecule contained two regions of homology to SSAV, separated by a 1.0 kbp nonhomologous region. This SSV-specific sequence was shown to be uniquely represented within the normal cellular DNA of diverse mammalian species, including human. Our results demonstrate that this primate transforming retrovirus arose in nature by recombination of a type C helper virus and a host cellular gene.

Cellular DNAs of different vertebrate species have been examined for homology with the cell-derived sequence of SSV, designated sis. Nucleotide sequences homologous to sis were detected at low copy number within cellular DNAs of species as diverse as human and quail. Using the molecular hybridization techniques, sis was shown to have originated from within the woolly monkey (Lagothrix spp.) genome. Thus, sis arose from a normal primate gene which is uniquely represented and highly conserved within vertebrate species.

We have recently cloned the human gene which is homologous to sis. The cloned human DNA contains 1.0 kbp of sis-related information and 7.0 kbp which is unrelated. Comparison of the molecular structure of the human gene with sis is nearly complete. We have also begun to analyze transcripts of human tumor cells for homology with sis or human sequences contiguous with sis-related information. Transfection experiments to determine whether this human gene possesses transforming activity are also in progress.

Significance to Biomedical Research and the Program of the Institute:

Highly infectious molecular clones of SSV provide valuable reagents which will be useful in investigating the mechanisms involved in cellular transformation induced by this primate sarcoma virus. Molecular clones of the SSV-related human gene provide the opportunity to determine whether its altered or enhanced expression is involved in naturally occurring human malignancies.

Proposed Course:

1. To continue detailed molecular and biologic analysis of the SSV-transforming gene.

2. To continue investigations of the involvement of sis and its human analogue in naturally occurring malignancies.

3. To begin efforts to analyze the translational products of the SSV genome using cloned SSV DNA as an aid.

Publications:

Robbins, K.C., Devare, S.G., and Aaronson, S.A.: Molecular cloning of integrated simian sarcoma virus: genome organization of infectious DNA clones. Proc. Natl. Acad. Sci. USA. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05168-01 LCMB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of the Human Cytochrome P-450 locus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. Swan Expert LCMB NCI

COOPERATING UNITS (if any)
Laboratory of Biochemistry, DCBD, NCI
Developmental Pharmacology Branch, NICHHD
Laboratory of Molecular Genetics, NICHHD

LAB/BRANCH
~~Carcinogenesis Intramural Program~~
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The murine Ah locus regulates de novo induction of liver microsomal cytochrome P_1-450 by polycyclic aromatic compounds such as 3-methylcholanthrene (MC). The 23S mRNA for P_1-450 has been shown to be induced by MC in Ah-responsive C57BL/6N mice. Using partially purified 23S mRNA from MC-induced mice, a double-stranded complementary DNA was synthesized and inserted into the Pst I site in pBR322 plasmid DNA. One clone which was positively identified as containing part of the P_1-450 gene had an 1100 bp insert. Labelling of isolated insert by nick-translation has allowed us to identify a closely related sequence in human placenta DNA. This human equivalent has now been cloned in the phage vector Charon 16A.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. To isolate and characterize the human cytochrome P_1-450 gene.

2. To ascertain the number and chromosomal location of the cytochrome P-450 genes in man.

3. To determine the complete DNA sequence of the human P_1-450 gene.

Methods Employed:

Messenger RNA isolated from 3-methylcholanthrene-treated C57BL/6N mice was purified by oligo dT-cellulose chromatography and sucrose gradient centrifugation. A double-stranded complementary DNA copy was synthesized using avian myeloblastosis virus reverse transcriptase and DNA polymerase I. Single-stranded ends were removed by S1 nuclease and oligo dC tails added by terminal deoxynucleotidyl transferase. These dC-tailed fragments were then inserted into pBR322 plasmid DNA which had been cut with Pst I restriction endonuclease and tailed with oligo dG. Seventy-two ampicillin-sensitive, tetracycline-resistant clones were analyzed by hybridization to [^{32}P] cDNA synthesized using mRNA from methylcholanthrene-treated C57BL/6N responder mice and DBA/2N non-responder mice. One clone (clone 47) was found which hybridized to responder, but not to non-responder cDNA. The insert in this clone was shown to be 1100 bp in length. In translation arrest experiments the cloned DNA was bound to diazobenzyloxymethyl (DBM) paper and used to bind mRNA from methylcholanthrene-induced C57BL/6N mice. The bound mRNA was shown immunologically to code for P_1-450 when DNA from clone 47 was bound to the DBM-paper but not when DNA from any of the other ampicillin-resistant clones was used.

The human P_1-450 gene has been identified by hybridization of nick-translated clone 47 DNA to Eco RI digested human placenta DNA. Human placenta DNA was first restricted by Eco RI endonuclease and separated on the basis of base composition by reverse phase chromatography on RPC-5. Each of twenty fractions from the RPC-5 column was then electrophoresed in a 1% agarose gel and transferred to DBM-paper. Hybridization of this filter to nick-translated clone 47 DNA under relaxed hybridization conditions showed the presence of a 4 kb sequence which has now been isolated and cloned in Charon 16A.

Major Findings:

1. The cytochrome P_1-450 gene has been synthesized on mRNA from methylcholanthrene-induced C57BL/6N mice and cloned in pBR322.

2. The cloned cDNA gene has been used as a probe to identify corresponding sequences in mouse and human genomic DNA.

3. The human equivalent to methylcholanthrene-induced P_1-450 has been isolated and shown to be 4 kb in size. It has been cloned in Charon 16A.

Significance to Biomedical Research and the Program of the Institute:

Cytochrome P-450 represents a group of NADPH- and/or NADH-dependent membrane-bound multicomponent enzyme systems in eukaryotic cells. In addition to basal (constitutive) forms of P-450 that metabolize normal body substrates such as steroids and biogenic amines, there are forms of P-450 that can be induced by drugs and other foreign chemicals. The general consensus of most laboratories has been that there are six or less forms of induced P-450, although it has been suggested that organisms may have the genetic capacity for the induction of hundreds or thousands of forms of P-450. Because of problems with detergent solubilization of microsomal membranes, the isolation of truly homogenous forms of P-450 has remained exceedingly difficult. Amino acid sequencing has been attempted, but the data are highly variable and inconsistent between laboratories--even among the first five or ten amino acids from the N-terminal end. Studies of the nucleotide sequence not only will complement any protein sequence data but also should provide important insight into the understanding of P-450 induction.

Proposed Course:

1. Determine how many P-450 genes comprise the Ah locus in human.

2. Determine the chromosomal location of this locus.

3. Study the sequences responsible for regulation of P-450 expression.

Publications:

Negishi, M., Swan, D.C., Enguist, L.W., and Nebert, D.W.: Isolation and characterization of a cloned DNA sequence associated with the murine Ah locus and a 3-methylcholanthrene-induced form of cytochrome P-450. Proc. Natl. Acad. Sci. USA 78: 800-804, 1981.

Leder, A., Swan, D., and Leder, P.: Alpha-like globin genes of the mouse have spread to three different chromosomes. Nature. (In press.)

Nebert, D.W., Negishi, M., Enquist, L.W., and Swan, D.C.: Use of recombinant DNA technology in the study of genetic differences in drug metabolism affecting individual risk of malignancy. Proceedings of ICN-UCLA Symposia on Molecular and Cellular Biology, 1981. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05169-01 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Characterization of Transforming Genes and Proteins of Abelson Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Yuasa	Visiting Fellow	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	P.E. Reddy	Visiting Scientist	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI
	J. Pierce	Staff Fellow	LCMB	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0
PROFESSIONAL: 1.0
OTHER: 0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Abelson virus transforms both fibroblasts and lymphoid cells, and has about 3.5 kbp src specific sequences. By isolating deletion mutants from the cloned Abelson virus DNA, transformation-specific regions are being determined.

The Bgl II 1.5 kbp fragment-deleted mutant did not transform fibroblasts. Another mutant, which deletes Bgl II 0.8 kbp fragment near 3' end of the genome, transformed both fibroblasts and lymphoid cells. These data show that 2.7 kbp DNA sequences, except 0.8 kbp Bgl II fragment, are enough for both types of transformation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. To identify whether transforming proteins of Abelson virus responsible for fibroblast and lymphoid cell transformation are the same or different.

2. To determine the transformation-specific regions of Abelson virus genome.

Methods Employed:

1. Making deletion mutants by modifying the cloned Abelson virus DNA with restriction enzymes.

2. Transfection of deleted genomes of Abelson virus DNA on cultured fibroblasts.

3. Rescue of Abelson virus by superinfection with helper MuLV on the transformed cells.

4. In vitro and in vivo transformation assay of lymphoid cells by the rescued viruses.

5. Protein analysis by immunoprecipitation and protein kinase assay.

Major Findings:

Abelson leukemia virus tranformed both fibroblasts and lymphoid cells and has about 3.5 kbp of src specific sequences. The protein coded by the viral genome has been shown to contain protein-kinase activity. In order to localize the genetic sequences needed for transforming function of this virus, several deletion mutants of this virus were constructed. Two such deletion mutants were derived by the removal of sequences between the Bgl II restriction sites. One such mutant lacked 1.5 kbp near the 5' end of the transforming region and the second mutant lacked 0.8 kbp of the sequences near the 3' end of the transforming region. The biological activity of these mutants was tested by transfection of DNA into NIH 3T3 cells. The results indicate:

1. The Bgl II 1.5 kbp fragment-deleted genomes of Abelson virus did not transform fibroblasts by DNA transfection.

2. The second deletion mutant, which lacked Bgl II 0.8 kbp fragment at the 3' end transformed fibroblasts.

3. Abelson virus could be rescued by helper MuLV from the cells transformed by 0.8 kbp fragment-deleted DNA. The rescued virus transformed both fibroblasts and lymphoid cells.

Significance to Biomedical Research and the Program of the Institute:

Abelson virus is the only known mammalian retrovirus which transforms both fibroblasts and lymphoid cells. It is very important to determine whether Abelson virus has two different transforming proteins or only one protein responsible for both types of transformation. Abelson virus is also very useful for analyzing the differentiation process of immunoglobulin producing lymphocytes, since Abelson virus specifically transforms pre-B cells *in vitro*.

Proposed Course:

1. Determine the exact transforming regions of Abelson virus genome for fibroblast and lymphoid cell transformation by isolating many deletion mutants.

2. Analyze the molecular weight and protein kinase activity of transforming proteins of deletion mutants.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05170-01 LCMB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Markers of Viral Transformation in Human Skin and Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Koh	Visiting Fellow	LCMB	NCI
Other:	J. S. Rhim	Research Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Viral Immunology Section

INSTITUTE AND LOCATION
NCI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to (1) establish cell lines of human skin and epithelial cells following infection with RNA and DNA viruses, and (2) study the markers of transformation in these cells in order to establish the criteria for the recognition of malignant transformation essential for complete development of in vitro models to study carcinogenesis.

PHS-6040
(Rev. 2-81)

Project Description

Objective:

To study the markers of viral transformation in human skin and epithelial cells.

Methods Employed:

Biological methods include cell cloning, cell aggregation and soft agar. Serum dependence and growth factor requirements of human skin and epithelial cells will be determined. For testing the oncogenicity of transformed cells, nude mice will be used.

Major Findings:

1. Various clonal lines of human skin fibroblasts infected by Ki-MSV and Ad12-SV40 viruses were established.

2. Clonal lines of human skin epithelial cells infected with Ad12-SV40 virus were established.

Significance to Biomedical Research and the Program of the Institute:

The establishment of criteria for the recognition of malignant transformation is essential for complete development of *in vitro* models to study carcinogenesis.

Proposed Course:

Characteristics of various clonal lines of viral transformed human skin and epithelial cells should allow us to determine the markers of transformation leading to neoplasia.

Publication:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05189-01 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies Define Viral Gene Products Associated with Murine Mammary Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Colcher	Microbiologist	LCMB	NCI
Others:	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
	Jeffrey Schlom	Section Chief	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies have been used to define the diversity of mammary tumor viral-coded gene products associated with murine mammary tumors. Monoclonal antibodies have been generated against disrupted mammary tumor viruses (MTVs) isolated from M. musculus, M. cervicolor and M. cookii. Antibodies directed against the gp36 external glycoprotein and p28 internal protein of these viruses demonstrated the presence of multiple epitopes that represent type, group and interspecies determinants. The monoclonals have also been used to distinguish six MTV isolates from each other, including both endogenous and exogenous viruses from the same strain, and a new virus isolate from BALB/c mice. Employing the immunoperoxidase technique, the monoclonal antibodies have been used to define a heterogeneity of expression of MTV gene products in primary mammary tumors of three Mus species. These studies have revealed that a given tumor-associated antigenic determinant may be expressed differentially in mammary tumors of two different species, among mammary tumors of the same species and, at times, in different areas of the same mammary tumor.

Project Description

Objectives and Historical Background: Murine mammary tumor viruses (MMTVs) have been isolated from a variety of strains of laboratory mice (*M. musculus*) with different incidences of mammary cancer. Horizontally transmitted MMTVs have readily been identified in the milk of mice which usually have a high incidence of "early" mammary tumors. In contrast, vertically transmitted MMTVs such as MMTV(C3Hf) have been associated with a lower incidence of mammary tumors that occur relatively late in life.

Radioimmunoassays (RIAs) have been developed for the MMTV external glycoproteins of 52,000d (gp52) and 36,000d (gp36), as well as the 28,000d (p28) major internal polypeptide. Type-specific RIAs have been used to distinguish the MMTVs from RIII and GR mice from the MMTV of C3H mice. Group-specific RIAs for the MMTV polypeptides (in which all MMTVs of *M. musculus* react) have also been used to examine milk and mammary tumors from other *Mus* species. Using these RIAs, a virus with several morphological and biochemical properties in common with MMTVs of *M. musculus* has been identified in the milk of *M. cervicolor*, and has been termed MC-MTV. This virus was then used as an immunogen and the resulting antisera were used to establish interspecies RIAs for MTV antigens. These RIAs have been used to identify a related virus, termed MCo-MTV, in milks of animals of the species *M. cookii*.

Antibodies to MMTV polypeptides have also been used to examine the immunocytochemical distribution of MMTV antigens in both primary and metastatic mammary tumors of *M. musculus*. The immunoperoxidase staining of the various mammary tumors still leaves open the question, however, as to whether the different patterns of staining observed are due to the expression of different antigenic determinants of MMTV or the differential expression of the same determinant. Studies to answer these questions necessitate the use of monoclonal antibodies to a given MMTV determinant.

It is the purpose of these studies to generate monoclonal antibodies to the major polypeptides of MTVs. These antibodies could then be used to further define type, group and interspecies reactivities among MTVs derived from strains of laboratory mice (*M. musculus*) commonly used in mammary cancer research. These antibodies could also be used to define the diversity of expression of individual MTV antigenic determinants in primary mammary tumors of various *Mus* strains and species and may also serve as a prototype for the detection of mammary tumor-associated antigens.

Major Findings: Mice and rats were immunized with disrupted MTVs from *M. musculus* (MMTV[C3H]), *M. cervicolor* (MC-MTV) and *M. cookii* (MCo-MTV). Standard hybridoma technology was employed to obtain monoclonal antibodies that were reactive with test immunogens.

Monoclonal antibodies were then characterized for class and subclass. One was an IgM and 13 others were various IgG subclasses. All the monoclonal antibodies bound selectively to disrupted MMTV(C3H) and to membrane enriched extracts of cells producing MMTV(C3H). All the monoclonal antibodies bound to membranes from both C3H and GR mammary tumor cell lines, and membranes of feline CrFK cells producing MMTV(C3H). No binding was observed, however, to membranes of C3H fibroblasts or the uninfected CrFK feline cell line. These findings provide evidence that the antigenic determinants bound by these antibodies are viral-mediated and most probably viral coded. The major MMTV(C3H) polypeptides, gp52, gp36 and p28, were purified using hydrophobic ion-exchange chromatography and molecular sieving. Using solid phase RIAs the various monoclonals were then characterized as to their polypeptide specificity. The monoclonals reacted specifically with either purified gp52, gp36, or p28.

The monoclonal antibodies that were generated against the type B retroviruses from *M. cervicolor* and *M. cookii* were tested for reactivity to retroviruses from a variety of species. Antibodies M1.1 (prepared from animals immunized with MC-MTV) and M3.1 (prepared from animals immunized against MCo-MTV) both reacted with MTVs from *M. cervicolor* and *M. musculus*. None of the monoclonal antibodies tested bound to MuLV from *M. musculus*, or the M432 retrovirus from *M. cervicolor*. The monoclonal antibodies showing interspecies reactivity for MTVs were then tested against a variety of type C and type D retroviruses of different species, and no binding was observed.

The various monoclonal antibodies then were tested for their ability to bind to various MMTVs from several strains of mice. The viruses were also propagated in feline cells to ensure that the reactivities observed were not directed against murine determinants. One monoclonal antibody bound to the highly oncogenic MMTVs from C3H and GR mice but not to the highly oncogenic MMTVs from RIII and A mice. This type specificity was confirmed by the reactivity of this antibody to purified gp36 from MMTV(C3H) but not to the purified gp36 from MMTV(RIII). This monoclonal was also able to distinguish between two MMTVs with moderate oncogenicity, i.e., it bound to the MMTV purified from BALB/c mice but not MMTV from C3HfC57BL mice. This monoclonal was also able to distinguish between the horizontally transmitted MMTV(C3H) and the vertically transmitted MMTV(C3Hf) of C3H mice.

The monoclonal antibodies generated were then used to distinguish a particular MMTV. In most cases the source of the MMTV could be identified using only one monoclonal antibody. For example, to distinguish MMTV(A) from other MMTVs it was only necessary to examine the reactivity with monoclonal antibody R3.1. MMTVs from six different strains of *M. musculus* could clearly be distinguished from each other by this method. Monoclonal antibodies have also been generated that react to all the MMTVs tested.

The immunoperoxidase technique was employed to determine if the monoclonal antibodies generated could be used to detect MTV antigenic determinants on tissue sections of primary and transplanted murine mammary tumors. Two major types of staining patterns were observed: apical staining, in which the immune reaction was concentrated at the periphery of lumens of acini, and focal staining, in which the immune reaction was detected as discrete intracytoplasmic foci. Two phenomena became apparent using these antibodies to monitor expression of a distinct antigenic determinant in tumor cells. The first was that a given antigenic determinant can be expressed in a different manner in two different mammary tumors. The second phenomenon was the quantitative heterogeneity in staining within a given mammary tumor, i.e., most mammary tumors tested from a variety of *Mus* strains and species presented some areas that were positive, and some that were negative, for expression of a given determinant.

The immunoperoxidase technique was also employed to further define interspecies reactivities of certain monoclonal antibodies. For example, one monoclonal antibody reacted significantly with sections of primary mammary tumors of M. musculus (C3H), M. cookii, and M. cervicolor. This antibody did not, however, react with sections of lactating mammary glands of C57BL mice, a strain devoid of MTV antigen expression in its milk. The immunoperoxidase technique has also proved useful in the detection of tumor-associated antigens in segments of tumors too small to be employed in other immunological assays.

Significance to Biomedical Research and Proposed Course: These studies involve the generation and characterization of the first repertoire of monoclonal antibodies to the major structural proteins of MMTVs. These antibodies have demonstrated the diversity of viral gene products of MMTVs from various mouse strains and provide a model for the study of well-defined mammary tumor-associated antigens. The use of the immunoperoxidase technique with monoclonal antibodies has revealed the heterogeneity of antigen expression in mammary tumors, i.e., that a single antigenic determinant may be expressed in a different manner not only among different mammary tumors but in different areas of the same mammary tumor. Furthermore, these studies have demonstrated a great diversity in the level of expression of a given antigenic determinant within a given primary mammary tumor. The monoclonal antibodies directed against various interspecies determinants of MTV gene products now also makes possible an evaluation of the association between viral gene products and mammary tumorigenesis in feral populations of species other than M. musculus, the laboratory mouse.

Publications:

Colcher, D., Horan Hand, P., Teramoto, Y. A., Wunderlich, D., and Schlom, J.: Use of monoclonal antibodies to define the diversity of mammary tumor viral gene products in virions and mammary tumors of the genus Mus. Cancer Res. 41: 1451-1459, 1980.

Schlom, J., Colcher, D., Drohan, W., Horan Hand, P., Howard, D., and Teramoto, Y. A.: Systematics of murine mammary tumor viruses. In Brennan, M., Rich, M. A., and McGrath, C. M. (Eds.): Breast Cancer Research: New Concepts in Etiology and Control. New York, Academic Press, Inc. 1980, pp. 149-171.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05190-01 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Monoclonal Antibodies Reactive with Human Mammary Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Colcher	Microbiologist	LCMB	NCI
Others:	Marianna Nuti	Fogarty Fellow	LCMB	NCI
	Daniela Stramignoni	Fogarty Fellow	LCMB	NCI
	Jeffrey Schlom	Section Chief	LCMB	NCI

COOPERATING UNITS (if any)
Sidney Farber Cancer Institute, Boston, Massachusetts
Dept. of Pathology, George Washington University, Washington, D. C.

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of these studies is to generate and characterize monoclonal antibodies that are reactive with human mammary tumor cells. Splenic lymphocytes of mice, immunized with membrane-enriched fractions of metastatic human mammary carcinoma tissues, were fused with murine myeloma cells. This resulted in the generation of hybridoma cultures secreting Igs reactive in solid phase radioimmunoassays with extracts of metastatic mammary carcinoma cells from involved livers, but not to extracts of apparently normal human tissues. Eleven monoclonal antibodies were chosen which demonstrated reactivities with human mammary tumor cells and not with apparently normal human mammary tissue. These monoclonals could be placed into at least five major groups based on their differential binding to the surface of various live human mammary tumor cells in culture, to extracts of mammary tumor tissues, or to tissue sections of mammary tumor cells employing the immunoperoxidase technique. Monoclonal antibodies of all five major groups demonstrated binding to human metastatic mammary carcinoma cells both in axillary lymph nodes and at distal sites.

Project Description

Objectives and Historical Background: Numerous investigators have reported the existence of human mammary tumor-associated antigens. These studies, all conducted with conventional hyperimmune polyclonal sera, however, were unfortunately hampered with regard to the heterogeneity of the antibody populations employed, and the amount of specific immunoglobulin that could be generated. Since the advent of hybridoma technology, monoclonal antibodies of predefined specificity and virtually unlimited quantity may now be generated against a variety of antigenic determinants present on normal and/or neoplastic cells. The rationale of these studies is to utilize extracts of human metastatic mammary tumor cells as immunogens in an attempt to generate and characterize monoclonal antibodies that are reactive with determinants that would be maintained on metastatic, as well as primary, human mammary carcinoma cells. Multiple assays using tumor cell extracts, tissue sections, and live cells in culture were employed to determine the reactivities of the monoclonal antibodies generated.

Major Findings: Mice were immunized with membrane-enriched fractions of human metastatic mammary carcinoma cells from either of two involved livers (designated Met 1 and Met 2). Spleens of immunized mice were fused with NS-1 myeloma cells to generate approximately 4,000 primary hybridoma cultures. Supernatant fluids from these cultures were screened in solid phase RIAs for the presence of immunoglobulin reactive with extracts of metastatic mammary tumor cells from involved livers and not reactive with similar extracts of apparently normal liver. Whereas many cultures demonstrated immunoglobulin reactive with all test antigens, 370 cultures contained immunoglobulin reactive only with the metastatic carcinoma cell extracts. Following passage and double cloning of these cultures, the monoclonal immunoglobulins from eleven hybridoma cell lines were chosen for further study. The isotypes of all eleven antibodies were determined; ten were IgG of various subclasses and one was an IgM. The primary screen for monoclonal antibodies reactive with human mammary carcinoma cells was a solid phase RIA employing cell extracts of two breast tumor metastases (Met 1 and Met 2) and apparently normal human liver as test antigens. The eleven monoclonal antibodies could immediately be divided into three major groups based on their differential reactivity to Met 1 vs. Met 2. Nine of the eleven monoclonals were reactive with both metastases. One monoclonal, however, was reactive only with Met 1, and another monoclonal was reactive only with Met 2. All eleven antibodies were negative when tested against similar extracts from normal human liver, a rhabdomyosarcoma cell line, a cell line derived from cultures of human milk, a mouse mammary tumor cell line, a mouse fibroblast cell line, a feline kidney cell line, and disrupted mouse mammary tumor virus and mouse leukemia virus. Two monoclonal antibodies were used as controls in all these studies which showed reactivity to all human cells tested. The solid phase RIA using cell extracts of metastatic breast tumor cells proved quite sensitive for the detection of test antigen. The assays routinely employed 5 ug of tissue extract, but titration experiments showed that as little as 0.3 ug of tissue extract could be used.

To further define the reactivities of the eleven monoclonal antibodies, and to determine if they bind cell surface antigens, each antibody was tested for binding to live cells in culture. Test cells included three established cell lines of human mammary carcinoma (BT-20, MCF-7, and ZR-75-1), several cell lines of other human tumors, and eleven cell lines established from apparently normal human tissues. The nine monoclonals grouped together on the basis of their binding to both metastatic cell extracts could be separated into three different groups on the basis of their differential binding to the surface of live mammary cells in culture. None of the antibodies bound to several sarcoma and melanoma cell lines tested. Some of the antibodies appeared to possess a "pancarcinoma" pattern of binding activity. Two of the monoclonals, on the other hand, did not react with the surface of any of the mammary tumor cell lines tested, but could be distinguished from the other nine monoclonals by their differential binding to cell extracts. None of the eleven monoclonal antibodies bound to any of the following cell lines derived from apparently normal human tissues: breast, uterus, skin, embryonic skin and kidney, and fetal lung, testis, thymus, bone marrow, and spleen. Control monoclonals, however, did bind all of these cells.

To further define the range of reactivity of each of the eleven monoclonal antibodies, the immunoperoxidase technique on tissue sections was employed. All the monoclonals reacted with mammary carcinoma cells of primary mammary carcinomas. A high degree of selective reactivity was observed with mammary tumor cells, and not with apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes of the breast with all eleven monoclonal antibodies.

Significance to Biomedical Research and Proposed Course: Monoclonal antibodies that are reactive with human mammary carcinoma cells may eventually be useful in the diagnosis, prognosis, and treatment of human breast cancer. Antibodies could be radioactively labeled for in-situ scanning to detect metastatic lesions in nodes of the internal mammary chain and at distal sites. Preliminary experiments will first be carried out employing a nude mouse model and transplanted human mammary tumors. Experiments are also under way to determine if the presence or absence of any one or combination of the antigenic determinants recognized by these monoclonal antibodies on tissue sections have any prognostic value, i.e. does it reflect the degree of differentiation or malignancy of a given mammary tumor cell population. These monoclonals may also serve a useful purpose in providing a marker for the transformed state in in-vitro carcinogenesis experimentation.

Publications:

Colcher, D., Horan Hand, P., Teramoto, Y. A., Wunderlich, D., and Schlom, J.: Use of monoclonal antibodies to define the diversity of mammary tumor viral gene products in virions and mammary tumors of the genus Mus. Cancer Res. 41: 145-1459, 1980.

Colcher, D., Horan Hand, P., Nuti, M., Schlom, J.: A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc. Natl. Acad. Sci. (USA) (In Press).

Nuti, M., Colcher, D., Horan Hand, P., Austin, F., and Schlom, J.: Generation and characterization of monoclonal antibodies reactive with human primary and metastatic mammary tumor cells. In Proceedings of the Conference on Radioimmunoassay. North Holland, Elsevier (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05191-01 LCMB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Generation of Human Monoclonal Antibodies from Lymph Nodes of Mastectomy Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
Others:	Jeffrey Schlom	Section Chief	LCMB	NCI

COOPERATING UNITS (if any)

Dept. of Surgery, George Washington University, School of Medicine, Washington, D.C.
Michigan Cancer Foundation, Detroit, Michigan

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lymphocytes from lymph nodes obtained at mastectomy in breast cancer patients have been fused with murine non-Ig producer myeloma cells to obtain human-mouse hybridoma cultures that synthesize human monoclonal antibodies. Immunoglobulin (Ig) production was stable in many of the cloned cultures through a 60-300 day observation period, with levels of human Ig synthesis ranging from 0.1-20ug/ml of supernatant fluid. Using the immunoperoxidase technique and tissue sections, several human IgM's demonstrated binding to human mammary tumor cells. One human IgM monoclonal antibody was used to discriminate between mammary carcinoma cells (from 55 of 59 patients) and normal mammary epithelial cells, stroma, or lymphocytes of the same breast. These studies demonstrate that stable clones of human-mouse hybridomas, secreting human Ig, can be generated using lymph nodes of mastectomy patients.

Project Description

Objectives: The objective of these studies is the generation of human monoclonal antibodies that are reactive with human mammary carcinoma cells. The rationale behind these studies is that axillary lymph nodes, draining breasts bearing a mammary tumor, may contain B-lymphocytes that are primed against antigens that may be shed from tumor cells. These human primed B-lymphocytes could then be fused with non-Ig secreting murine myeloma cells to form human mouse hybridomas that secrete human Ig. These hybridoma cultures could then be cloned in an attempt to obtain stable hybridoma cultures synthesizing human monoclonal antibodies.

Major Findings:

Generation and Characterization of Human-Mouse Hybridoma Cultures. To date, over 1,400 microtiter wells have been seeded with fusion products of murine NS-1 myeloma cells (non-Ig secreting) and lymphocytes from 16 patients. Of these, 301 human-mouse hybridoma cultures have been successfully propagated. All nodes were from patients with infiltrating duct or lobular adenocarcinoma. Replicating human-mouse hybridoma cultures were obtained from lymph node segments of 81% of patients. Hybridoma cultures were first tested for the synthesis of human IgG or IgM 14-28 days after fusion. Of the 301 replicating hybridoma cultures, 52 (17%) synthesized either human IgG or human IgM. The duration of human Ig synthesis ranged from 14 to at least 300 days. Twenty-three of the 52 cultures (44%) continued to synthesize human Ig through the 61 to 300 days of observation.

The level of human IgG or human IgM production was measured by liquid immunobead competition and solid-phase radioimmunoassays. The titers observed were comparable to those detected in our laboratory and by others with both mouse-mouse and mouse-rat hybridomas. Twenty-seven of 52 (52%) human-mouse hybridoma cultures synthesized human Ig at levels equal to or greater than 1 ug/ml. Seven of these cultures synthesized human Ig at levels of 10 to 20 ug/ml. Many of the human-mouse hybridoma cultures were unstable for Ig production. For example, only 7 of 21 primary clones of hybridomas from one patient previously shown to be positive for Ig production on day 71, were positive when assayed on day 94. Upon recloning the three clones highest for Ig synthesis, however, 126 of 129 secondary clones remained positive for human Ig synthesis through the 300 day observation period.

Characterization of Human Igs. Several assays were used to determine if the Igs secreted by the human-mouse hybridoma cultures were indeed human and not murine. These included: (a) immunobead liquid competition radioimmunoassays, (b) solid phase linker radioimmunoassays, and (c) Ouchterlony double-diffusion. The immunobead and solid-phase radioimmunoassays could readily distinguish between human and mouse Igs and between human IgG and human IgM. Mouse Igs did not react in these assays. Two additional assays were used to determine if the human-mouse hybridoma supernatant fluids displayed any evidence of murine Igs; both gave negative results. A solid phase linker radioimmunoassay that could detect 30 ng of murine Ig also failed to detect any murine Ig in human monoclonal antibody supernatant fluids.

Some of the human-mouse hybridoma supernatant fluids were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and found to possess complete Ig, i.e., both heavy and light chains. For example, an IgM preparation used in immunoperoxidase studies demonstrated bands with apparent molecular weights of approximately 75,000 and 22,000, consistent with the apparent molecular weights of heavy and light chains, respectively, of IgM. This human monoclonal IgM preparation was also subjected to velocity sedimentation analysis and demonstrated a sedimentation coefficient of approximately 19S, corresponding to pentameric IgM.

Immunoreactivity of Human Monoclonal Antibodies. To define the immunologic reactivities of the human Igs secreted by human-mouse hybridoma cultures, supernatant fluids were analyzed on tissue sections of human mammary tumors using the immunoperoxidase technique. Cells reactive with the monoclonal antibody would show the characteristic reddish-brown insoluble diaminobenzidine reaction product in contrast to the blue hematoxylin counterstain. Supernatant fluids from several hybridoma cultures from five patients showed some degree of immunoperoxidase reactivity with mammary carcinoma cells in sections of their own mammary tumors. One IgM monoclonal antibody (MBE6 from patient MB) from double cloned cultures was chosen for further study.

Monoclonal antibody MBE6 was first tested by immunoperoxidase for reactivity with tissue sections of the primary breast tumor mass (infiltrating duct carcinoma) of patient MB and demonstrated marked cytoplasmic staining in most of the mammary tumor cells. Differences in both intensity of staining and percentage of cells stained were observed in various areas of the primary tumor mass. Antibody MBE6 could distinguish clearly between malignant mammary cells and "non-malignant" mammary epithelium or stromal cells. Antibody MBE6 was then tested for its ability to detect metastatic mammary carcinoma cells in the lymph nodes of patient MB and could clearly distinguish between metastatic breast cells and lymphocytes. A variation in intensity of staining was also observed within a given population of metastatic cells of a given lymph node.

This same monoclonal antibody was also tested for its ability to bind to mammary carcinoma cells of patients other than MB. In the vast majority of these patients, MBE6 could be used to distinguish between malignant and "non-malignant" mammary epithelial cells. A few of the "normal" mammary epithelial cells in areas adjacent to primary tumor cells of a few patients also showed staining with MBE6. Some of the benign breast tumors tested also showed staining with MBE6. This latter staining, however, usually was much lighter and more diffuse than the staining of mammary carcinoma cells. Preliminary studies also indicate a cross reactivity between MBE6 and cells of selected non-breast adenocarcinomas, such as a bronchial alveolar carcinoma and a medullary carcinoma of the thyroid. MBE6 did not stain smooth muscle, arteries, veins, and nerve bundles of various breasts and did not react with normal colon, thyroid, lung or cartilage. Monoclonal antibody MBE6 was also tested for its ability to detect metastatic mammary carcinoma cells in lymph nodes of patients other than MB and could readily discriminate between mammary carcinoma cells and adjacent lymphocytes or stroma in metastatic lymph nodes of breast cancer patients. Differences in the intensity and pattern of staining were observed in various areas of a given mammary tumor. Some tumor cells demonstrated intense staining throughout the cytoplasm; such heavily stained cells were often immediately adjacent to unstained tumor cells. Alternatively, some tumor cells showed a more localized perinuclear stain. To date, monoclonal MBE6 has demonstrated reactions with mammary carcinoma cells of 55 of 59 patients tested.

Significance to Biomedical Research and Proposed Course: The availability of monoclonal antibodies that are reactive with human mammary tumor cells may ultimately have utility in the diagnosis, prognosis, and treatment of human breast cancer, and may be very useful in the study of the immune response to human mammary cancer cells. If monoclonal antibodies are ever used clinically, human monoclonal antibodies may have the advantage over murine monoclonal antibodies in that one would anticipate a reduced immune response to human immunoglobulin. The human monoclonal antibodies thus far generated and characterized have been of the IgM isotype. Further studies are underway to determine if useful human IgG monoclonal antibodies can be generated and characterized.

Publications:

Colcher, D., Horan Hand, P., Teramoto, Y. A., Wunderlich, D., and Schlom, J.: Use of monoclonal antibodies to define the diversity of mammary tumor viral gene products in virions and mammary tumors of the genus *Mus*. *Cancer Res.* 41: 1451-1459, 1980.

Schlom, J., Wunderlich, D., and Teramoto, Y. A.: Generation of human monoclonal antibodies reactive with human mammary carcinoma cell antigens. *Proc. Natl. Acad. Sci. (USA)* 77: 6841-6845, 1980.

Teramoto, Y. A., Mariani, R., Wunderlich, D., and Schlom, J.: The immunohistochemical reactivity of a human monoclonal antibody with tissue sections of human mammary tumors. *Cancer* (In Press).

Wunderlich, D., Teramoto, Y. A., Alford, C., and Schlom, J.: Generation of human monoclonal antibodies using lymphocytes from axillary lymph nodes of mastectomy patients. *Eur. J. Cancer* (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05199-01 LCMB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Molecular and Immunologic Studies of Murine Mammary Tumorigenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey Schlom	Section Chief	LCMB	NCI
Others:	Robert Callahan	Microbiologist	LCMB	NCI
	William Drohan	Sr. Staff Fellow	LCMB	NCI
	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
	Michael Potter	Section Chief	LCBGY	NCI

COOPERATING UNITS (if any)

Michigan Cancer Foundation, Detroit, Michigan
Baylor College of Medicine, Houston, Texas

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have investigated the relationship between mammary cancer and the presence of specific endogenous and infectious mouse mammary tumor virus (MMTV) genetic elements in high, moderate, and low tumor incidence mouse strains. In the high incidence GR stain, a single endogenous provirus has been identified which segregates with the susceptibility to mammary cancer. A novel MMTV variant has been isolated from the low incidence strain BALB/c that is unique both biochemically and immunologically from all existing MMTVs. Noncoordinate gene expression has also been identified in preneoplastic lesions and mammary tumors of the BALB/c and other mouse strains. A high mammary tumor incidence outbred colony of Mus cervicolor, derived from a feral population, has been established. Interspecies radioimmunoassays and monoclonal antibodies have identified, in this colony, a virus related to, but distinct from, MTVs of laboratory mice. These molecular and immunologic studies are delineating the broad diversity of genes and gene products associated with mammary tumors of a given genus and species.

Project Description

Background and Rationale: For several decades MMTV has been thought to be involved in inducing spontaneous mammary cancer in some strains of inbred mice. Hybridization experiments performed in this laboratory and others have made it clear that several different MMTV variants may be involved in this process. The large number of copies of MMTV present in normal mouse cells have made it difficult to precisely define the mechanism by which MMTV transforms mammary epithelial cells. We have attempted to use restriction endonuclease analysis to develop molecular markers which distinguish among endogenous and infectious variant of MMTV, and thus provide us with a tool to begin to sort out the role different MMTVs play in the neoplastic transformation of murine mammary epithelial cells. We have attempted to correlate the presence of specific MMTV proviral variants with the incidence of mammary tumors in mouse strains with a low, moderate or high incidence of mammary cancer as well as in feral, outbred populations of the genus Mus. The data suggest that several different mechanisms are involved in the transformation of mammary epithelium.

Major Findings: C3H/StWi mice have a low incidence of spontaneous mammary tumors. In spite of the fact that four distinct endogenous MMTV proviruses have been identified in this strain, only 7 out of 441 breeding females developed mammary tumors. To determine which, if any, of the endogenous proviruses was involved in inducing the mammary tumors, high molecular weight DNA from normal C3H/StWi tissue was digested with specific restriction endonucleases and the MMTV-specific DNA fragments identified by hybridization to MMTV-cDNA. Our studies have shown that if MMTV is involved in the induction of spontaneous or chemically induced C3H/StWi mammary tumors it does so by another mechanism than the simple amplification of pre-existing endogenous MMTV genes. It should be noted that this is the first system in which spontaneous murine mammary tumors have been shown to arise without the presence of amplified MMTV proviral information.

Although the mammary tumor incidence in most strains of BALB/c mice is low, over 50% of the breeding females of the BALB/cV strain develop mammary tumors by the age of 10 months. To determine if any alteration in the MMTV proviral information of these mice was involved in this dramatic increase in tumor incidence, we examined the MMTV proviral DNA content of both normal and tumor tissue of BALB/cV mice. In addition to endogenous MMTV-specific bands found in all organs of BALB/cV mice, we identified two additional MMTV -specific restriction fragments in DNA from BALB/cV mammary tumors. These fragments appeared to contain the genetic information for an infectious MMTV provirus that could be distinguished from any of the endogenous BALB/cV proviruses. To date the additional MMTV proviral information found in mammary tumors of BALB/cV mice can be distinguished from any endogenous or exogenous MMTV variant reported thus far. In fact, we have been able to isolate an infectious virus from the BALB/cV strain of mice. This new MMTV variant has been shown to be oncogenic for BALB/c mice and also immunologically distinguishable (using monoclonal antibodies) from other infectious variants of MMTV.

The GR strain of mice are interesting in that over 95% of breeding females develop mammary cancer before 1 year of age. Traditionally the MMTV has been thought to be involved in the induction of mammary tumors in GR mice. However, the mechanism by which MMTV might induce mammary cancer in the strain is unclear since both liquid hybridization studies and restriction endonuclease analysis has identified five endogenous MMTV proviruses in normal GR tissues. To identify which endogenous GR provirus(es) is involved in the induction of mammary cancer, crosses were set up between GR mice and C57BL mice in which the incidence of mammary cancer is 0%. In BC1 females [C57Blx(C57BlxGR)] mammary tumor incidence segregated as a single Mendelian unit in that 50% of the animals developed mammary cancer. We have developed three molecular markers for a single endogenous GR provirus which segregates as a single Mendelian unit with the occurrence of mammary cancer in these mice. The markers are (1) MMTV specific Sac 1 restriction fragments of 6.2, 2.2, and 0.9 Kbp, (2) MMTV specific Bgl II restriction fragment of 4.3 Kbp and (3) a subset of nucleic acid sequences of the RNA genome of the highly oncogenic MMTV(C3H) virus called tumor associated sequences. Thus we have identified a single endogenous GR provirus which appears to be involved in the induction of mammary cancer in GR mice.

We have recently completed a retrospective study of the incidences of neoplasias in the various colonies of mice. With one exception each colony had a tumor incidence of 1 to 2%. In contrast, the M. cervicolor popaeus Tak colony has a 10% incidence of neoplasias. Significantly over half of these tumors were of the mammary gland. Protein extracts of the mammary tumors were tested for the expression of endogenous retroviruses (type C-I, type C-II, M432, and a type B retrovirus termed MC-MTV) in competition radioimmunoassays; eighteen of 21 tumors contained detectable levels of MC-MTV-associated proteins. Normal tissues from the same mice were negative in this assay. Similar conclusions were reached with immunoperoxidase assays using antisera prepared against MC-MTV. Type C-1 associated proteins were present in 5/21 tumors while type C-II and M432 related proteins were not detected. In addition, preliminary results of restriction enzyme analysis of cellular DNA from some of the mammary tumors suggests that the MC-MTV genome may be amplified in a manner similar to that observed in M. musculus C3H mammary tumors. These results suggest that MC-MTV may play an etiological role in mammary tumors of this species.

Significance to Biomedical Research and Proposed Course: It is now apparent that there are a wide variety of variants of MMTVs differing in their nucleic acid sequence, antigenic specificity, site of integration, biological activity and mode of transmission. This thus complicates the task of precisely defining which variant is actually involved in tumorigenesis in a given population. Restriction endonuclease analysis and the Southern blot technique now allow us to distinguish between most of the endogenous and infectious MMTV proviruses. We now have the potential to assign specific biological activities to specific genetic loci, such as the newly acquired MMTV proviral information in BALB/cV mammary tumors or the gene endogenous to GR mice. We now can thus attempt to define at a molecular level, the involvement of specific MMTV genetic elements in the transformation process in both inbred and naturally occurring feral populations.

Publications:

Colcher, D., Horan Hand, P., Teramoto, Y. A., Wunderlich, D., and Schlom, J.: Use of monoclonal antibodies to define the diversity of mammary tumor viral gene products in virions and mammary tumors of the genus *Mus*. *Cancer Res*. 41: 1451-1459, 1980.

Drohan, W., Cardiff, R. D., Lund, J. K., and Schlom, J.: Correlation between the detection of specific mouse mammary tumor proviral sequences and the presence of pulmonary metastases in mice bearing spontaneous mammary tumors. *Cancer Res.* 40: 2316-2322, 1980.

Drohan, W., Young, J., and Schlom, J.: Correlation between the development of murine mammary cancer and the segregation of endogenous genes. In Fields, B., Jaenisch, R., and Fox, C. F. (Eds.): *Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology*. New York, Academic Press, 1980, pp. 177-185.

Horan Hand, P., Teramoto, Y. A., Callahan, R., and Schlom, J.: Interspecies radioimmunoassays for the major internal protein of mammary tumor viruses. *Virology* 101: 61-71, 1980.

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Lopez, D. M., Sigel, M. M., Charyulu, V., Ortiz-Muniz, G., Schlom, J., and Lozzio, B. B.: Cell-mediated immunity to MMTV antigen(s): Its relevance to host defenses against mammary tumors. In Brennan, M., Rich, M. A., and McGrath, C. M. (Eds.): *Breast Cancer Research: New Concepts in Etiology and Control*. New York, Academic Press, Inc., 1980, pp. 387-406.

Schlom, J.: Type-B and type-D retroviruses. In Stephenson, J. (Ed.): *Molecular Biology and RNA Tumor Viruses*, New York, Academic Press, Inc., 1980, pp. 447-484.

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Schlom, J., Drohan, W., Teramoto, Y. A., Young, J. M., and Horan Hand, P.: Diversity of mammary tumor viral genes and gene products in rodent species. In Essex, M., Todaro, G., and zur Hausen, H. (Eds.): *Cold Spring Harbor Conferences on Cell Proliferation - Viruses in Naturally Occurring Cancer*. New York, Cold Spring Harbor Laboratory, 1980, pp. 1115-1132.

Teramoto, Y. A., Horan Hand, P., Callahan, R., and Schlom, J.: Detection of novel murine mammary tumor viruses by interspecies immunoassays. *J. Natl. Cancer Inst*. 64: 967-975, 1980.

Teramoto, Y. A., Medina, D., McGrath, C., and Schlom, J.: Noncordinate expression of murine mammary tumor virus gene products. *Virology* 107: 345-353, 1980.

Callahan, R., Hogg, E., Sass, B., Teramoto, Y. A., Todaro, G. J., and Schlom, J.: A feral population of *M. cervicolor popaeus* with a high incidence of spontaneous mammary tumors. *J. Natl. Cancer Inst*. (In Press).

Drohan, W., Teramoto, Y. A., Medina, D., and Schlom, J.: Isolation and characterization of a new MMTV variant from BALB/c mice. *Virology* (In Press).

CONTRACT NARRATIVE
LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1981

HAZLETON LABORATORIES AMERICA, INC. (N01-CP-01017)

Title: Support Services for the Laboratory of Cellular and Molecular Biology

Contractor's Project Director: Mr. James Gargus

Project Officer (NCI): Dr. Stuart A. Aaronson

Objectives: The purpose of the contract is to provide support services for research conducted by the LCMB.

Methods Employed and Performance: The contractor performs the following tasks: (a) purification of retrovirus proteins, (b) performance of radioimmunoassays, (c) analysis of translational products synthesized by virus-infected cells, (d) detection of RNA-dependent DNA-polymerase, (3) isolation of cellular and viral nucleic acids, (f) preparation of viral nucleic acid probes, (g) performance of molecular hybridization assays, (h) restriction endonuclease analysis of viral and cellular DNAs, (i) biological assays for RNA tumor viruses, (j) growth of cells and viruses, (k) maintenance of animals, and (l) preparation and quality control of tissue culture medium.

Significance to Biomedical Research and Proposed Course: This program provides essential support services for research of the LCMB aimed at determining the etiology of naturally occurring cancers, elucidation of mechanisms of transformation, and the development of approaches capable of prevention of spontaneous and virus-induced tumors (see Annual Reports).

Date Contract Initiated: June 1980

Current Annual Level: $705,000

MELOY LABORATORIES (N01-CP-01018)

Title: Support Services for the Experimental Oncology Section,
Laboratory of Cellular and Molecular Biology

Contractor's Project Director: Fred Rogusky

Project Officer (NCI): Dr. Jeffrey Schlom

Objective: The purpose of this contract is to supply support services for the Experimental Oncology Section of the Laboratory of Cellular and Molecular Biology.

Methods Employed and Performance: The contractor performs the following tasks: (a) purification of cellular and viral proteins, (b) preparation of viral nucleic acid probes, (c) isolation of cellular DNA and RNA, (d) biological assays for mammary tumor viruses, (e) preparation, cloning, and storage of hybridoma cell lines, (f) assay of monoclonal antibodies, (g) propagation of cells in tissue culture, (h) provide facilities for housing mice, rats, and rabbits, and breeding of mice, (i) provide tissue culture media and sterility testing, (j) decontamination, cleaning, and sterilization of glassware.

Significance to Biomedical Research and Proposed Course: To continue support services for the Experimental Oncology Section, Laboratory of Cellular and Molecular Biology. The Experimental Oncology Section has made significant progress in areas that may be applicable in the diagnosis, prognosis and treatment of several human neoplasms (see Annual Reports).

Date Contract Initiated: November 20, 1980

Current Annual Level: $480,055

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SUMMARY REPORT

LABORATORY OF CHEMOPREVENTION

October 1, 1980 through September 30, 1981

Our Laboratory has continued with its program of research, dealing with biological, biochemical, and pharmacological aspects of the ability of retinoids to control epithelial cell differentiation and to prevent the development of epithelial cancer. These studies have a two-fold purpose: from a basic scientific viewpoint, we wish to understand the mechanism of action of retinoids as completely as possible, while from a pragmatic, clinical viewpoint, we would like to see this field developed in support of the practical use of retinoids for prevention of cancer in men and women at high risk for development of malignancy. There is by now an extensive experimental literature that retinoids can be used effectively, both *in vitro* and *in vivo*, to suppress the phenotypic expression of malignancy caused by a variety of carcinogens, and further research in this area needs to be pursued.

Several different aspects of retinoid biology, biochemistry, and pharmacology have been studied in our Laboratory. In the area of biology, we have studied the mechanism of the interaction between retinoids and new growth factors (transforming growth factors, TGF's) first isolated by Todaro and De Larco. These low molecular weight, highly stable polypeptides confer properties of the transformed phenotype (growth in soft agar, release from density-dependent cell growth) on normal cells and their action can be blocked by very small doses of retinoids *in vitro*. In collaborative studies with Drs. Todaro and De Larco, we are purifying the growth factors and examining the molecular and cellular basis whereby retinoids block their transforming action. The animal resource at IIT Research Institute in Chicago, headed by Dr. Richard Moon, has been a critical facility for the productive investigation of the ability of retinoids to prevent the development of cancer of the breast and bladder in experimental animals, as well as to conduct initial investigations of the ability of retinoids to alter or modify proliferative states associated with other human diseases. In a recent review article, we have summarized a set of experimental data which suggest that retinoids may also be of definite relevance to the study and prevention of several important proliferative diseases other than cancer.

The problem of the isolation and characterization of transforming polypeptide growth factors (TGF's) is occupying an increasing amount of attention within our Laboratory. Previously, we had shown that TGF's can be isolated from a variety of epithelial and mesenchymal tumors of murine, chicken, and human origin, caused either by chemicals or viruses, or of spontaneous origin. All of these TGF's are acid-stable, low molecular weight materials, that will be the subject of future attempts at amino acid sequencing once they are purified to homogeneity. New methods to achieve this desired purification have already been developed in our Laboratory during the past year. Based on the known properties of these TGF's, we will attempt to design new synthetic polypeptide inhibitors, which should represent a new class of chemopreventive agents.

Our efforts to purify these factors to homogeneity continue. Using the TGF's from MSV-transformed cells as a guide, high-pressure liquid chromatography (HPLC) systems have been developed which give high resolution and good recovery of biological activity. It is anticipated that three or four sequential HPLC steps will yield a homogeneous preparation. During the course of this purification, it has been found that the soft agar colony-forming activity of the TGF's of both neoplastic and non-neoplastic tissues is markedly potentiated by nanogram quantities of another mitogen, epidermal growth factor (EGF). This finding has greatly increased the sensitivity of our assay. Whether, upon final purification, these TGF's will show an absolute requirement for EGF for the expression of biological activity remains to be demonstrated.

TGF's have also been detected in all human tumor cell lines investigated to date. Several of the cell lines which display the highest activity have been selected for further purification studies. These include a cervical carcinoma cell line (HeLa), a rhabdomyosarcoma (A673), a fibrosarcoma (HT1080) and a melanoma (A2058) cell line. In addition, TGF-like activity has also been found in normal human placenta. Initial purification of the polypeptides from these sources has been undertaken using an acid-ethanol extraction procedure to isolate the acid-soluble proteins followed by gel permeation chromatography of these proteins on a Bio-Gel P30 column. Two peaks of transforming activity in the molecular weight range of 18,000 to 20,000 and 6,000 to 10,000 have been eluted from this column. Each of these peaks, after reverse-phase high-pressure liquid chromatography, can be further separated into additional peaks of transforming activity. The activity of several of these peaks can be markedly enhanced in the presence of epidermal growth factor. Additional methods, including ion exchange chromatography and polyacrylamide gel electrophoresis, are currently being investigated for further purification of these various human polypeptides.

The structural and functional relationships between EGF and TGF's are being studied as a means to design peptide inhibitors of malignant transformation, which would represent a totally new type of chemopreventive agent. The finding that EGF is a strong potentiator of TGF activity is an important one. New peptide studies have therefore been started in a new research program in our Laboratory. These studies are attempting to define structure-function relationships of the EGF molecule, through synthetic and chemical modification techniques, primarily to gain information for the rational design of effective inhibitors. These inhibitors hopefully will be used to study the mechanism of action of the TGF's and could ultimately have clinical applications in the prevention or treatment of proliferative diseases. The chemical structures of the TGF's will also be determined as they are purified and structure-function studies on these peptides will then begin.

In actual experimental work, seven C-terminal synthetic fragments of EGF have been prepared by solid phase methodology to date and are being examined in a variety of assays for receptor binding and biological activity (agonist and antagonist). In addition, several modifications to the native EGF molecular have been made, and more are planned, to identify residues or regions of specific importance. Efforts to prepare larger synthetic fragments by solid phase, as well as fragment condensation, techniques are under current investigation. Substituting peptide linkages which are susceptible to enzymatic cleavage with linkages which are resistant to such cleavage is one way of altering the activity of polypeptide hormones. Such selective changes might allow one to pinpoint the positions in the polypeptide chain which are responsible for binding and/or biological activity. These analogs might conceivably possess antagonist activity. The class of compounds known as pseudodipeptides are those components in which the amide linkage has been replaced by a thioether. We have therefore started a project to synthesize a number of such thioethers with the object of incorporating them in selective spots in the EGF molcular. Thus far, two pseudodipeptides have been prepared and characterized.

The involvement of our Laboratory in the field of peptide chemistry is new. Staff members are still being recruited and equipment is still being obtained for necessary experimental work. At the time of writing this report, we have only been involved in many aspects of actual laboratory work, particularly in the area of peptide inhibitors, for less than 6 months. However, we believe that this will turn out to be an important area of research and will generate significant new pharmacological agents for prevention of cancer in the near future.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05051-03 LC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Isolation of Polypeptide Transforming Factors from Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Anita B. Roberts	Senior Staff Fellow	LC	NCI
	Mario A. Anzano	Visiting Fellow	LC	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4.0	PROFESSIONAL: 2.0	OTHER: 2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop methods for the extraction, isolation, and characterization of polypeptides with the property of conferring the transformed phenotype on normal indicator cells. These purified transforming growth factors (TGFs) will then form the basis for investigation of the mechanism of transformation and, more specifically, for the development of protein antagonists to these TGFs. Central to the investigation is the purification and characterization of the sarcoma growth factors (SGFs) found upon direct extraction of murine-sarcoma virus-transformed cells grown in culture. Once isolated, the generality of this SGF will then be investigated by comparing its properties to those of TGFs isolated from a variety of tumor cells of non-viral origin, as well as to those of polypeptides with similar characteristics isolated from non-neoplastic tissues.

Project Description

Objectives: The ultimate goal of this research is to determine the complete amino acid sequence of a transforming growth factor (TGF) and then to use this as a basis for the design of antagonists to these polypeptides. Once the protein is purified, it is our intention to investigate the mechanism of action of these TGF's in the carcinogenic transformation of cells, as well as the mechanism of their inhibition by potential synthetic polypeptide antagonists or natural antagonists such as the retinoids, which are known to have anti-promoter activity.

Methods Employed: Classical methods of protein purification and new methods based on reverse-phase high-pressure liquid chromatography have been employed. These include dialysis, solvent precipitation, the use of sizing gels and ion-exchange columns, as well as methods based on the electrophoretic properties of these polypeptides.

An assay for these TGF's, based on their ability to cause a normal anchorage-dependent indicator cell to grow in an anchorage-independent manner (as assessed by growth in semi-solid agar medium), has been set up in our laboratory and is being used routinely to monitor the purification of these TGF's.

Major Findings: Polypeptides characterized by their ability to confer a transformed phenotype on an untransformed indicator cell have been isolated directly from tumor cells growing both in culture and in the animal, using an acid-ethanol extraction procedure. Assay of these polypeptides is based on their ability to induce normal rat kidney fibroblasts to form colonies in soft agar. Peptides from murine sarcoma virus-transformed mouse 3T3 cells grown in culture had the highest specific activity in this assay; peptides from sarcomas produced from these cells, or from chemically induced transplantable bladder carcinomas of mice, were one-third as active, and peptides from a chemically induced rat tracheal carcinoma had only one-tenth the activity. Treatment with either trypsin or dithiothreitol destroyed the activity of all of these materials. Thus, these intracellular polypeptides from both virally and chemically transformed cells have properties similar to those described for the sarcoma growth factors (SGFs) previously isolated by De Larco and Todaro (NCI) from the conditioned medium of sarcoma virus-transformed mouse 3T3 cells, suggesting the definition of a new class of transforming growth factors (TGFs) common to tumor cells of different origin. The transforming peptides from the cultured sarcoma virus-infected cells were separated by gel filtration into two fractions of apparent molecular weight 7,000 and 10,000. The major fraction at 7000 M.W. represented approximately 0.1 percent of the original cell protein and had a specific activity 50-fold that of the original acid-ethanol extract.

More recently, it has been found that acid-ethanol extracts of non-neoplastic tissues of both the mouse and the steer also contain polypeptides which share with the TGF's of neoplastic tissues the property of inducing the transformed phenotype on normal indicator cells. The soft-agar colony-forming activity of the TGF's of both neoplastic and non-neoplastic tissues has been found to be markedly potentiated by nanogram quantities of another mitogen, epidermal growth factor (EGF). The assayable level of transforming activity of the acid-ethanol extracts of various tissues can be related in part to the levels of endogenous EGF. Assayed in the presence of optimal concentrations of EGF, the specific colony-forming activity of extracts from both neoplastic and non-neoplastic sources is comparable. By several criteria, including chemical stability, sensitivity to proteolytic enzymes and elution position on sizing gels, the TGF's of non-neoplastic tissues appear similar to those derived from neoplastic cells. Bovine tissues are currently being explored as inexpensive sources of large quantities of TGFs.

Significance to Biomedical Research and the Program of the Institute: The discovery of TGFs which act as direct effectors of phenotypic transformation, together with the discovery that retinoids can block the effect of SGF on cells, offers a unique opportunity to explore the mechanism of control of the carcinogenic process. Eventually, it is hoped that a better approach to the chemoprevention of carcinogenesis will develop from the results of these investigations. In addition, a more immediate application of these TGF's may arise from collaborative investigations currently underway concerning the possible role of these factors in wound healing.

Proposed Course : Future work will center around the purification of the intracellular SGF from murine-sarcoma virus-infected mouse 3T3 cells as well as of TGFs of non-neoplastic tissues, most notably the mouse or bovine salivary gland. Once purified, it is hoped that similar methods can be used to purify other TGFs. Attempts will be made to produce anti-serum to these TGFs to facilitate their quantitation in various neoplastic and non-neoplastic tissues. In addition, using radioiodinated TGFs, we hope to explore the cellular controls on the synthesis and excretion of these TGFs, the nature of the membrane receptors for these TGFs, as well as the possibility of blocking the expression of the biological activity of these TGFs by the use of synthetic or natural antagonists. The latter approach may reveal the physiologic role of those TGFs in non-neoplastic tissues.

Publications:

Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., De Larco, J.E., and Todaro, G.J.: Transforming growth factors; Isolation of polypeptides from virally and chemically transformed cells by acid-ethanol extraction. *Proc. Nat. Acad. Sci.* 77: 3494-3498, 1980.

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., and Sporn, M.B.: Transforming growth factors: Isolation of polypeptides from non-neoplastic tissues of the adult mouse. *Proc. Nat. Acad. Sci.*, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05116-02 LC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Characterization of Polypeptide Transforming Factors from Human Tumor Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Charles A. Frolik	Chemist	LC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Acid stable polypeptides have been isolated from human tumor cells and from normal human tissues. These proteins are able to produce a reversible morphological transformation of normal rat kidney fibroblast cells that allows these cells to grow in a soft agar medium where normal cell growth is prevented. It is the purpose of this project to characterize these transforming growth factors and to ascertain the role these factors may play in the process of carcinogenesis. Initial investigation will concentrate on the purification and characterization of these transforming growth factors. Once these proteins have been characterized, analogs will be synthesized and tested for their ability to inhibit the transforming activity of the natural peptide and therefore, possibly, inhibit the carcinogenic process itself. Finally, the mechanism whereby these factors interact with the cell to cause phenotypic cell transformation will be investigated in order to gain a greater understanding of the process of carcinogenesis.

Project Description

Objectives: The purpose of this study is to explore new methods that may be useful in the chemoprevention of cancer. Initially, special emphasis will be placed on the isolation and characterization of acid stable polypeptide transforming growth factors (TGFs) found in various human tumor cells and in normal human tissue. Once characterized, TGF analogs will be synthesized and tested for their ability to block the action of TGF in causing phenotypic cell transformation.

Methods Employed: TGF is extracted from various samples using an acid-ethanol procedure developed by Dr. Anita Roberts, in our Laboratory, and is based on the insulin isolation method of Davoren. Once extracted, the peptides are purified further by gel permeation chromatography and by reverse-phase high-pressure liquid chromatography. Ion exchange chromatography and polyacrylamide gel electrophoresis are additional methods being investigated for their possible usefulness in the purification scheme. Transforming activity is determined by a soft agar growth assay, and the number and the size of the cell colonies obtained are measured using an Omnicon image analysis system.

Major Findings: TGF has been detected in all human tumor cell lines investigated to date. Several cell lines which display the highest activity have been selected for further purification studies. These include an intestinal tumor line, HeLa, and a rhabdomyosarcoma (A673), fibrosarcoma (HT1080) and a melanoma (A2058) cell line. Chromatography of the acid-ethanol extract of these cells on a BioGel P30 gel permeation column yields two peaks that display transforming activity. One peak is in the 18,000 to 20,000 weight range while the second peak is from 6,000 to 10,000. Each of these peaks, after high-pressure liquid chromatography on a reverse-phase column, can be further separated into additional peaks of transforming activity. The activity of several of these peaks can be markedly enhanced when epidermal growth factor is included in the soft agar assay. Additional methods are currently being investigated for further purification of these various polypeptides.

Significance to Biomedical Research and the Program of the Institute: Through a knowledge of the physical structure of TGF and its mode of action in causing phenotypic transformation, it is anticipated that a sound approach to the chemoprevention of carcinogenesis will be able to be developed.

Proposed Course: In the future, work will center around the purification and characterization of the transforming growth factors and could possibly include, as well, initial investigations into the mode of action of TGF in causing a reversible morphological transformation of normal cells.

Publications:

None. Project recently initiated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05209-01 LC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Synthetic and Biological Studies on Epidermal and Transforming Growth Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Chester A. Meyers	Expert	LC NCI
PI:	Akira Komoriya	Staff Fellow	LC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A group of transforming growth factors (TGF's) have been identified in both normal and transformed cells from a variety of tissue types. These factors, which are effectors of malignant phenotypic transformation, appear to be peptides possibly related to epidermal growth factor (EGF), and EGF markedly enhances the action of some TGF's. This study will attempt to define structure-function relationships of the EGF, and utimately the TGF molecules, through synthetic and chemical modification techniques, primarily to gain information for the rational design of effective inhibitors. Seven C-terminal synthetic fragments of EGF have been prepared by solid phase methodology to date and are being examined in a variety of assays for receptor binding and biological activity (agonist and antagonist). In addition, several modifications to the native molecule have been made and, more are planned, to identify residues or regions of specific importance. Efforts to prepare larger synthetic fragments by solid-phase, as well as fragment condensation techniques, are under current investigation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Chemical studies of EGF have to date been severely limited, presumably resulting from a combination of factors: 1) a lack of availability of large quantities of pure EGF; 2) lack of a well-defined biological assay which is specific for EGF (most assays in use are generally applicable to a vast array of mitogens), and; 3) until recently, an important role for EGF had not been recognized,and therefore no immediate use for chemical analogs having altered biological actions could be envisioned.

The objectives of the project are to first determine the regions of the EGF molecule responsible for receptor binding and biological activity in a variety of assay systems already developed in this laboratory. This would provide information needed for a rational approach to the design of synthetic peptide analogs which would be effective antagonists to the native peptide. These analogs would be used to investigate the manner in which TGF's cause cellular transformation; they may also have potential as therapeutic agents in the prevention or treatment of cancer and other proliferative diseases.

Methods Employed and Major Findings: The project embodies a broad program of chemical synthesis, modification, sequencing, analysis, chromatographic separations and biological testing. As a part of preparative scale isolation of TGF's by A. Roberts, large amounts of purified EGF are collected and used by us for studies in chemical modification, synthesis, and biological assays. In addition to the standard assays for general mitogens and specific binding assays for EGF, we are able to routinely test the peptides in a biological assay where EGF specifically enhances the transforming capability of TGF.

Peptide synthesis is primarily done by state-of-the-art solid-phase methodology and we are also developing methods for both conventional and enzymatic condensation of synthetic and native fragments. Purification of synthetic peptides is accomplished by a combination of gel filtration, ion exchange, partition, and reversed-phase high-performance liquid chromatographic techniques.

Seven synthetic fragments and analogs of EGF have already been prepared and two modifications to the native molecule have been accomplished. Early testing of some of these compounds in receptor-binding and mitogenic assays suggest that the C-terminal region of EGF is necessary for full binding and biological activity, and there is some evidence that the tryptophyl residues may be replaced by Phe without deleterious effects.

To design analogs that are refractory to enzymatic degradation while retaining as many native functional groups as possible, pseudo amino acids of tryptic and chymatryptic substrates are being synthesized (see report of N. Acton Z01 CP 05210-01 LC). If the degradation of EGF is an obligatory step for expression of its biological activity, then pseudo amino acid analogs may be effective inhibitors. To date, two pseudodipeptides have been synthesized.

Significance to Biomedical Research and the Program of the Institute: This work is expected to produce a major tool, namely, an antagonist to EGF, which can be used to probe the mechanisms by which cellular proliferation and transformation occur. This should broaden our understanding of cancer and several other proliferative diseases, In addition, these molecules, and related TGF analogs, have potential as clinical drugs in the treatment or prevention of such diseases.

Proposed Course: To continue as described above.

Publications:

None. Project recently initiated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05210-01 LC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Synthesis and Characterization of Some Pseudodipeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy Acton Roth	Expert	LC	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Substituting peptide linkages which are susceptible to enzymatic cleavage with linkages which are resistant to such cleavage is one way of altering the activity of polypeptide hormones. Such selective changes might allow one to pinpoint the positions in the polypeptide chain which are responsible for binding and/or biological activity. These analogs might conceivably possess antagonist activity. The class of compounds known as pseudodipeptides are those compounds in which the amide linkage has been replaced by a thioether. Some of these compounds were synthesized by Yankeelov, and coworkers (J. Org. Chem., 43, 1623 (1978)), who suggested that they might have a backbone conformation similar to that of the peptide analog while being resistant to hydrolysis. We have started a project of synthesis of a number of these thioethers with the object of incorporating them in selective spots in the epidermal growth factor molecule. Thus far, three pseudodipeptides, as well as diasteriomers of two of them have been prepared and characterized.

Project Description

Objectives: The purpose of this study is to synthesize peptide analogs which, when incorporated into the epidermal growth factor molecule, might alter its activity in such a way as to confer inhibitory properties, and might also help to elucidate positions in the molecule which are important for binding and/or biological activity.

Methods Employed: The synthetic methods are based on Yankeelov, and coworkers (J. Org. Chem. 43, 1623 (1978)) and involve stereospecific synthesis of a tosyl derivative of one amino acid and a thiol derivative of a second amino acid, followed by coupling of these two derivatives in a nucleophilic displacement to give the pseudodipeptide.

Major Findings: Three pseudodipeptides, as well as diasteriomers of two of them, have been prepared and characterized. In addition, several intermediates required to prepare a number of other dipeptide analogs have been synthesized.

Significance to Biomedical Research and the Program of the Institute: Several polypeptides in the group of growth factors are thought to play a role in malignancy. By studying chemically altered growth factors, it is hoped that a better understanding of their mode of action could be achieved. In addition, if any of these chemically altered polypeptides exhibit inhibitory activity, an approach may be opened to chemoprevention of carcinogenesis.

Proposed Course: In the future, several more pseudodipeptides will be synthesized and characterized in an appropriate form for insertion via solid phase synthesis into a synthetic growth hormone or a fragment of a growth hormone.

Publications:

None. Project recently initiated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05230-01 LC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Isolation and Characterization of Transforming Growth Factors from Chicken Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael B. Sporn	Chief	LC NCI

COOPERATING UNITS (if any)
Laboratory of Molecular Biology, NCI

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop methods for the extraction, isolation, and characterization of polypeptides with the property of conferring the transformed phenotype on normal indicator cells. These purified transforming growth factors (TGF's) will then form the basis for investigation of the mechanism of transformation and, more specifically, for the development of protein antagonists to these TGF's. Central to the investigation is the purification and characterization of the TGF's found in chicken cells transformed by Rous sarcoma virus. Such tissues are obtained from birds inoculated with RSV or from cells transformed by the virus in culture. TGF's have also been obtained from normal chick embryos.

Project Description

Objectives: The goal of this project is to isolate, purify, and characterize a set of transforming growth factors (TGF's) from chicken tumor cells that have been transformed by Rous sarcoma virus (RSV). This system has been chosen because the RSV system is perhaps the best understood of all transformation systems, from a mechanistic viewpoint, and the role of TGF's in transformation can therefore be studied in context with a great deal of other key information. Since TGF's undoubtedly have a role in non-neoplastic tissues, parallel studies are also being conducted in normal chick embryos. Once TGF's have been isolated and sequenced, it is intended to develop specific competitive peptide antagonists, with the ultimate goal of using such agents for chemoprevention of cancer.

Methods Employed: Classical methods of protein purification and new methods based on reverse-phase high-pressure liquid chromatography have been employed. These include dialysis, solvent precipitation, the use of sizing gels and ion-exchange columns, as well as methods based on the electrophoretic properties of these polypeptides.

An assay for these TGF's based on their ability to cause a normal anchorage-dependent indicator cell to grow in an anchorage-independent manner (as assessed by gowth in semi-solid agar medium), has been set up in our Laboratory and is being used routinely to monitor the purification of these TGF's.

Major Findings: Transforming growth factors have been isolated from normal 4 day old chick embryos and from fibrosarcomas induced in newborn chicks by Rous sarcoma virus (RSV). TGF's have been extracted from tissue with 0.2 M HCl-73% ethanol and have been assayed by their effects on NRK cells in a soft agar assay. TGF's have been further purified by gel exclusion chromatography on Bio-Gel P-60 using 1 M acetic acid as the eluant. An apparent MW range of 6,000 - 12,000 was found which differentiates them from the RSV src gene product, ppSrc60. Further purification has been achieved using high-pressure liquid chromatography. A reversed phase Waters uBondapak alkyl phenyl column was used with an acetonitrile-water-0.1% trifluoroacetic acid system. TGF's are stable at pH 2 both for 48 hours at 37° and for 3 min at 100°, while the activity is destroyed by dithiothreitol. Purification of these polypeptides from kilogram quantities of tissue is currently in progress.

Significance to Biomedical Research and the Program of the Institute: The discovery of TGF's which act as direct effectors of phenotypic transformation, together with the discovery that retinoids can block the effect of SGF on cells, offers a unique opportunity to explore the mechanism of control of the carcinogenic process. Eventually, it is hoped that a better approach to the chemoprevention of carcinogenesis will develop from the results of these investigations.

Proposed Course: To continue purification and characterization of TGF's and to develop specific peptide inhibitors to be used for chemoprevention.

Publications:

Sporn, M.B., Newton, D.D., Roberts, A.B., De Larco, J.L., and Todaro, G.J.: Retinoids and suppression of the effects of polypeptide transforming factors - a new molecular approach to chemoprevention of cancer. In Sartorelli, A.C., Bertino, J.R., and Lazo, J.S. (Eds.): Molecular Action and Targets for Cancer Chemotherapeutic Agents. New York, Academic Press, Inc., 1981, pp 541-554.

Sporn, M.B.: Retinoids: New developments in their mechanism of action as related to control of proliferative diseases. In Orfanos, C.E. et al. (Eds.): Retinoids. Germany, Springer-Verlag, in press.

Sporn, M.B., and Harris, E.D., Jr.: Proliferative diseases. Am. Journal Med., in press.

Sporn, M.B., and Newton, D.L.: Retinoids and chemoprevention of cancer. In Zedeck, M., and Lipkin, M. (Eds.): Inhibition of Tumor Induction and Development. New York, Plenum Press, in press.

CONTRACT NARRATIVE
LABORATORY OF CHEMOPREVENTION
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1981

IIT RESEARCH INSTITUTE (N01-CP-15742)

Title: Resource for Long-Term Animal Experiments to Study Prevention of Cancer by Retinoids and Related Materials.

Contractor's Project Director: Dr. Richard C. Moon

Project Officer (NCI): Dr. Michael B. Sporn

Objectives: The major objective of this contract is to provide the Laboratory of Chemoprevention, NCI, with an animal resource for evaluation of the chemopreventive activity of retinoids, natural and synthetic derivatives of vitamin A, and other related chemopreventive compounds in experimental models for cellular differentiation and tumorigenesis of the breast, lung, sebaceous glands and vascular system.

Major Findings: Mammary Cancer. A single intravenous administration of N-methyl-N-nitrosourea (MNU) has been shown to induce mammary cancers in female Sprague-Dawley rats in a dose-related manner with no acute toxicity. MNU-induced mammary cancers invade locally and metastasize to distant sites. A synergistic interaction was found between N-(4-hydroxyphenyl)retinamide (HPR) and bilateral ovariectomy in the inhibition of mammary carcinogenesis induced by MNU or 7,12-dimethylbenz(a)anthracene (DMBA). By contrast, although both agents inhibited MNU-induced mammary carcinogenesis, no synergism in cancer inhibition was noted between HPR and MVE-2, an inducer of interferon synthesis. A study is currently underway to assess the efficacy of 4-HPR, alone and in combination with selenium, in the inhibition of DMBA-induced mammary tumors in female C57BL/6 x DBA-2-F_1 (BDF) mice.
Urinary Bladder Cancer. Dose-response parameters have been defined for the induction of transitional cell carcinomas in male BDF mice by intragastric administration of the carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (OH-BBN). The retinoids N-(2-hydroxyethyl)retinamide, 13-*cis*-(2-hydroxyethyl)-retinamide, and N-(5-tetrazolyl)retinamide were found to have approximately equal activity in bladder cancer prevention, while 13-*cis*-(5-tetrazolyl)-retinamide was inactive. Animal studies to test the efficacy of N-butyl retinamide, 13-*cis*-butyl-retinamide, N-(4-hydroxybutyl)retinamide, and 13-*cis*-(4-hydroxybutyl) retinamide have been completed; histopathological analysis of tissues from this study is in progress. Similarly, while the animal study to test for interactions between HPR and MVE-2 in the inhibition of urinary bladder carcinogenesis has been completed, histopathology is ongoing.
Vascular Proliferative Lesions. The temporal relationship between cholesterol accumulation, cellular proliferation, and gross lesion appearance in the aorta and brachiocephalic arteries of male S.E.A. Japanese quail has been determined. Pilot studies have been conducted to determine the tolerance of Japanese quail to retinoid-supplemented diets, and dose-response and time-response parameters ti cholesterol-induced lesion development have been defined. A pilot study has indicated that 13-*cis*-retinoic acid may possess antiatherogenic activity in

quail fed an atherogenic diet. Studies currently are in progress to determine the influence of 13-cis-retinoic acid on cell proliferation, cholesterol deposition, and atherosclerotic lesion development in quail fed an atherogenic diet supplemented with 0.125% cholesterol and 0.0625% cholic acid.

Sebaceous Gland Differentiation. The effect of 13-cis retinoic acid, N-(4-hydroxyphenyl)retinamide and the trimethylmethoxyphenyl analog of retinoic acid ethyl ester (RO 10-9359) on cellular differentiation of a modified sebaceous gland of the hamster (flank organ) was initiated. Histologic evaluation of the flank organ is in progress.

Significance to Biomedical Research and the Program of the Institute:

Studies performed under this contract have led to the development of new models for the safe but rapid induction of highly proliferative lesions and cancers in animals. These models have not only provided suitable systems for the evaluation of chemopreventive activity of retinoids, but also provided techniques by which other retinoids and chemopreventive compounds can be evaluated at the cellular level. The data obtained from the evaluation of retinoids in various epithelial cancer models will hopefully lead to the clinical application of these compounds for prevention of cancer in groups at high risk.

Proposed Course: Newly synthesized retinoids and other chemopreventive agents will be evaluated for prevention of respiratory, breast, urinary bladder, and esophageal cancer as well as determining the antiproliferative effect of these compounds in the above animal models.

Date Contract Initiated: January 18, 1981

Current Annual Level: $704,000

Publications:

Becci, P.J., Thompson, H.J., Grubbs, C.J., and Moon, R.C.: Histogenesis and dose dependency of 1-methyl-1-nitrosourea-induced carcinoma in a localized area of the hamster trachea. J. Natl. Cancer Inst. 64: 1135-1140, 1980.

Mehta, R.G., Cerny, W.L., and Moon, R.C.: Distribution of retinoic acid binding proteins in normal and neoplastic mammary tissues. Cancer Res. 40: 47-49, 1980.

Thompson, H.J., Becci, P.J., Moon, R.C., Sporn, M.B., Newton, D.C., Brown, C.C., Nurrenbach, A., and Paust, J.: Inhibition of 1-methyl-1-nitrosourea-induced mammary carcinogenesis in the rat by the retinoid axerophthene. Arzneimettel Forschung/Drug Research 30: 1127-1129, 1980.

Becci, P.J., Thompson, H.J., Strum, J.M., Brown, C.C., Sporn, M.B., and Moon, R.C.: N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder cancer in C57BL/6 x DBA2-F_1 mice, a useful model for study of chemoprevention of cancer with retinoids. *Cancer Res*. 41: 927-932, 1981.

Grubbs, C.J., Becci, P.J., Thompson, H.J., and Moon, R.C.: Carcinogenicity of 1-methyl-1-nitrosourea and 1-ethyl-1-nitrosourea when applied to a localized area of the hamster trachea. *J. Natl. Cancer Inst*. 66: 961-965, 1981.

McCormick, D.L., Adamowski,, C.B., Fiks, A., and Moon, R.C.: Lifetime dose-response relationships for mammary tumor induction by a single administration of N-methyl-N-nitrosourea. *Cancer Res*. 41: 1690-1694, 1981.

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Moon, R.C., and Mehta, R.G.: Retinoid binding in normal and neoplastic mammary tissue. In Leavitt, W.L. (Ed): *Hormones and Cancer*. New York, Plenum Press, in press.

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SUMMARY REPORT

LABORATORY OF EXPERIMENTAL PATHOLOGY

October 1, 1980 through September 30, 1981

The Laboratory of Experimental Pathology plans, develops and implements a research program on the experimental pathology of carcinogenesis, involving (1) development, characterization and evaluation of experimental models of human cancer using in vivo and in vitro carcinogenesis methods; (2) experimental study of the pathogenesis of cancer in human tissues; (3) morphological and biochemical studies on chemical carcinogenesis and its inhibition in selected animal, tissue and cellular models; (4) research on the pathogenesis of cancer related to perinatal exposures; and (5) studies of biochemical mechanisms involved in the expression of the transformed state.

Reorganization: Until the Summer of 1981, the Laboratory consisted of the Office of the Chief and four Sections: "Differentiation Control", "Human Tissue Studies", "In Vitro Pathogenesis" and "Perinatal Carcinogenesis."

The Laboratory started in 1970 as the Experimental Pathology Branch in the NCI Carcinogenesis Program and was renamed Laboratory of Experimental Pathology in the Carcinogenesis Intramural Program in 1977. The Laboratory was established by Dr. U. Saffiotti, then Associate Scientific Director for Carcinogenesis, to provide a program focus for research on biological models for chemical carcinogenesis, correlating experimental animal organ models as well as tissue and cell culture models with the corresponding human pathology.

The Chiefs of the Branch/Laboratory were: U. Saffiotti, M.D. (Acting, 1970-71), R. R. Bates, M.D. (1971-73), J. M. Rice, Ph.D. (Acting 1973-74) and U. Saffiotti, M.D. (1974 to the present). The Sections were originally a Bioassay Section (R. R. Bates, M.D., Head), a Carcinogen Screening Section (J. H. Weisburger, Ph.D., Head) and a Histopathology Unit (R. R. Bates, M.D., Head). Two larger programs developed from these groups and later became independent: the Bioassay Program (established as the Carcinogen Bioassay and Program Resources Branch in 1973, evolved as the Carcinogenesis Testing Program in 1976 and merged into the National Toxicology Program in 1980) and the Carcinogen Metabolism and Toxicology Branch (established in 1973, E. K. Weisburger, Chief). In 1973 the Experimental Pathology Branch was expanded to include the following components: In Vitro Pathogenesis Section (S. H. Yuspa, M.D., Head), Perinatal Carcinogenesis Section (J. M. Rice, Ph.D., Head), Digestive System Carcinogenesis Section (R. R. Bates, M. D., Head, J. M. Rice, Ph.D., Acting Head; terminated, 1975), Endocrine Carcinogenesis Section (R. R. Bates, M.D., Head; D. Janss, Ph.D., Acting Head; terminated 1975), a Histopathology Unit (D. G. Kaufman, M.D., Head; terminated 1975) and an Ultrastructural Unit (C. C. Harris, M.D., Head; terminated 1974). In F.Y. 1974 a new Human Tissue Studies Section was established (C. C. Harris, M.D., Head) and the Tumor Pathology Section (R. A. Squire, D.V.M., Ph.D., Head) was transferred to the Branch from the Office of the Associate Director. In 1975 the Differentiation Control Section (L. M. De Luca, Ph.D., Head) was transferred to the Branch from the Lung Cancer Branch. With the 1976 reorganization that separated the Bioassay Program from the research programs, the Tumor Pathology Section was transferred to the Bioassay Program and Dr. Squire, who had been Associate Chief of the Experimental Pathology Branch (1974-76) became Associate Director for Carcinogenesis Bioassays. Dr. Saffiotti, who had been Associate Director for Carcinogenesis (1968-76) and held the function of Chief of the Experimental Pathology Branch in a dual capacity, took full-time responsibility for the Experimental Pathology Branch in 1976.

From inception to 1977-78, the staff of the Experimental Pathology Branch contributed extensively to the development, coordination and monitoring of the NCI Carcinogenesis Collaborative Program, funded mostly through contracts and interagency agreements. In particular, the Experimental Pathology staff was largely responsible for the scientific direction of the Biological Models Segment and significantly contributed to the Bioassay Segment, to the Lung Cancer Segment and to the Information and Resources Segment. In those years, the Experimental Pathology staff also collaborated closely with the Office of the Associate Director for Carcinogenesis and its Units on Program and Data Analysis, Lung Cancer, and Epidemiologic Pathology, as well as with staff in the other Laboratories and Branches of the Program. With the separation of extramural from intramural activities, ca. 1978, the Laboratory concentrated its functions only on intramural research, with the additional support of resource contracts. The staff also contributed to the collaborative research programs of NCI with NIOSH and EPA.

The first decade of activity resulted in considerable scientific advances and in the development of several new research methods, especially in the areas of tissue and cell cultures, carcinogen-tissue interactions, radioimmunoassays and biochemical mechanisms. This progress resulted in an intensive growth of research efforts, supported in part by some increases in staff positions, and by temporary and visiting appointments. The total space available to the Laboratory, however, did not expand in many years while new equipment and tissue culture facilities took increasingly more space. This situation resulted in considerable crowding and prevented further development of several research projects. The research accomplishments of the Laboratory and the severe restrictions imposed by space limitations were recognized by the site visit team from the Board of Scientific Counselors in March, 1979. The staff strived to make the best out of an increasingly difficult situation in order to maintain a high degree of productivity.

The present reorganization is finally providing a long-awaited solution to these problems. A considerable amount of new space was made available at the NCI Frederick Cancer Research Center. The scientific accomplishments of the senior staff of the Laboratory towards progress in chemical carcinogenesis research, which resulted in wide national and international reputation, are well recognized in the current reorganization. Three new independent Laboratories are being established from the sections in the Laboratory of Experimental Pathology, and the Laboratory will receive new resources to develop a new research program. This reorganization will therefore result in the following components:

- Laboratory of Experimental Pathology (Chief: U. Saffiotti, M.D.) (relocated in Building 560 at Frederick) with two new Sections on Respiratory Carcinogenesis and on Tissue Culture, including current programs in the Office of the Chief.

- Laboratory of Comparative Carcinogenesis (Chief: J. M. Rice, Ph.D.) (relocated in Building 538 at Frederick) including current programs of the Perinatal Carcinogenesis Section.

- Laboratory of Human Carcinogenesis (Chief: C. C. Harris, M.D.) including current programs of the Human Tissue Studies Section.

- Laboratory of Cellular Carcinogenesis and Tumor Promotion (Chief: S. H. Yuspa, M.D.) including current programs of the In Vitro Pathogenesis Section and of the Differentiation Control Section.

Each of the new Laboratories will have more space and resources and will constitute a new point of departure for further progress in different but related areas in the growing field of experimental pathology research in chemical and physical carcinogenesis.

This Annual Report concludes the present phase of activity of the Laboratory of Experimental Pathology.

Summary Report: The main focus of investigations in this Laboratory has been on the study of the pathogenesis of chemically induced neoplastic disease using morphological and biochemical approaches at all levels of biological organization, including human tissues, animal models, organ and cell cultures as well as molecular interactions. Particular emphasis was given to studies of chemical carcinogenesis in lining epithelia, which are the tissues of origin of most human cancers.

There is a fundamental need to relate the process of carcinogenesis to the specific characteristics of the tissues from which the induced tumors originate. Experimental chemical carcinogenesis is the result of chemical-biological interactions resulting in pathologic responses typical of the different tissues and cells of origin. Human cancer is characterized by a similarly wide variety of pathologic response patterns.

Until the last decade, our understanding of the process of carcinogenesis derived mostly from studies in animals and animal tissues. The development of methods for comparative evaluations of carcinogenesis mechanisms in various tissues and in various species, including the human, by in vivo and in vitro systems, has provided a much more relevant, in depth approach to the crucial problem of interpreting research findings in terms of human cancer pathogenesis. One important outcome of such investigations is a much more versatile and more selective approach to the assessment of carcinogenic risks for humans on the basis of experimental studies.

Numerous animal models for major forms of human cancer, especially those of epithelial origin, were developed in recent years. Work from this Laboratory contributed to the establishment and investigation of biological models for cancers of the epidermis, respiratory tract, digestive tract, pancreas, liver, kidney, mammary gland, endocrine system, central and peripheral nervous system, and others. These models became the tools for studying the pathogenesis of major types of cancer. Such studies have rapidly advanced our understanding of the cells of origin and the pathogenesis of major types of cancer, through combined morphological and biochemical methods in the study of the target tissues, and correlation at the organ, tissue, cellular and molecular levels.

A broad range of experimental pathology methods are being used for the study of chemical carcinogenesis, focusing on the sequence of events leading from the activation of carcinogens and their interaction with cellular macromolecules to the induction of a transformed state and to its expression in cells, tissues and the whole organism. Areas of special emphasis that unify different experimental approaches in the Laboratory include (a) the study of carcinogenesis in epithelial tissues, (b) the correlation of different levels of biological organization and of different species including humans, (c) the study of mechanisms that modify the carcinogenic response (inhibition, promotion, synergism), (d) the relationship of cell differentiation to carcinogenesis, and (e) the identification of markers of the interaction of carcinogens with target cells. This comprehensive approach to selected problems of cancer pathogenesis is designed to provide knowledge directly relevant to the understanding of the causation of human cancers and their prevention.

In the last decade, a sequence of studies was undertaken for each of several target tissues of major human cancers, such as skin, lung, colon, pancreas, esophagus, liver, kidney and others. These studies included all or part of the following steps: the observation of human pathology; the development of animal models for *in vivo* chemical induction of the specific tumor type; the development of organ explant cultures from the animal model; the development of organ culture methods for the corresponding human target tissue; the development of cell culture methods and cloning procedures for the target cells of animal and human tissues (including the use of chemically defined, serum-free media); identification of the cellular distribution, metabolic activation, binding and interaction of carcinogens in the target animal and human cells and the patterns of DNA repair; the identification of specific reaction products of carcinogens with target macromolecules, particularly through specific new immunoassay techniques; the quantitative variability of carcinogen-cell interactions; the development of methods for the induction by chemical carcinogens of neoplastic or pre-neoplastic changes in the target cells *in vitro* in both animal and human systems; the characterization of changes induced by carcinogens in such cells and the identification of altered markers or specific gene products; the correlation of carcinogenic events with stages of cell differentiation, cell replication and with prenatal and postnatal development; the role of biological modifiers, inhibitors, synergists and promoters in different biological models of carcinogenesis; and the correlation of mutagenic with carcinogenic events.

A much closer correlation has emerged between animal and human tissue responses to carcinogens than was previously known. More insight has been gained on species differences in the metabolic handling of various carcinogens and on the conditions that determine tissue responses, such as age of prenatal or postnatal development, role of enzyme inducers, combined effects of carcinogens and role of inhibitors or promoters. The establishment of culture systems for the experimental study of human cell responses to carcinogens has brought this whole effort to bear directly on our understanding of the human pathology of cancer induction. The advances in the elucidation of the mechanisms of carcinogenesis, of initiation, promotion, enhancement and inhibition, that have been made in the course of these studies, have been continuously evaluated in the light of concurrent progress in related studies.

The establishment of four Laboratories, with their new resources, out of the tightly packed, highly productive core of the Laboratory of Experimental Pathology, opens up a new period that promises the development of exciting research towards a better understanding and eventual prevention of human cancer induction.

A summary of the current activities and accomplishments of the Laboratory is given in the following reports.

OFFICE OF THE CHIEF - In addition to overall coordination of the Laboratory, the following activities were contributed by the research staff of this Office.

Criteria for the identification and evaluation of carcinogenic agents and for risk assessment were analyzed and defined; documentation was assembled and reviewed on carcinogenic effects of environmental chemicals and potential occupational carcinogens; specific fields of environmental carcinogenesis research were reviewed, with particular consideration for occupational carcinogens.

A model was developed for the consideration of multiple concurrent factors in the etiology of cancers in a human population.

Review and advice were contributed to the Occupational Safety and Health Administration in the preparation of the OSHA candidate list of selected chemicals considered for regulation as potential occupational carcinogens.

Other collaboration was given to the Environmental Protection Agency (EPA), National Institute on Occupational Safety and Health (NIOSH), Consumer Product Safety Commission (CPSC) and other federal agencies.

Extensive bibliographic documentation was assembled on dose-response relationships and on synergistic effects in carcinogenesis studies.

Laboratory studies were continued on dose-response relationships of carcinogens in mutagenesis and cell transformation assays and on the effects of concurrent exposure to multiple carcinogens. Using the Ames Salmonella assay, with or without metabolic activation, several combinations of carcinogens were studied in a large range of dose levels; studies using low levels of exposure showed clear examples of additive, synergistic and inhibitory interactions in mutagenesis: polycyclic aromatic hydrocarbons were mutually inhibitory, aromatic amines were mostly additive with examples of inhibitory and synergistic combinations, while a group of structurally different carcinogens was found to act synergistically.

Neoplastic transformation in a mouse fibroblast cell line, BALB/c 3T3 clone A31-1-1, was used for studies on dose-response and combined effects of carcinogens parallel to those for mutagenesis. Optimal test conditions and relative levels of transforming activity were determined for metabolically different types of carcinogens in this system. Studies on the role of cell density in the expression of transformation were initiated.

DIFFERENTIATION CONTROL SECTION: 1) Studies the biochemical mode of action of vitamin A and its derivatives, the retinoids, in controlling and modulating the expression of the normal and neoplastically transformed state, 2) conducts investigations of the metabolism of retinol and retinoic acid in normal and transformed cells, and 3) studies cell-surface glycoproteins involved in cell adhesion.

Advances have been made in two research areas: the basic area of the mechanism of action of vitamin A in glycoprotein synthesis and the area of the effect of retinoid treatment on cell surface receptors for specific polypeptide growth factors.

Mechanism of action of vitamin A. The work of this laboratory has demonstrated the molecular involvement of phosphorylated vitamin A in the following reactions:

1. Retinylphosphate + guanosine diphosphate-mannose ⟶ mannosylretinylphosphate + guanosinediphosphate.

2. Mannosylretinylphosphate + membrane acceptors $\longrightarrow$ mannosylated acceptors + retinylphosphate.

The first reaction has been shown to take place with endogenous and exogenous retinylphosphate, thus demonstrating that mammalian membranes contain both the substrate as well as the enzyme for this reaction. The Km for GDP-mannose was shown to be below the concentration of this substrate in physiological conditions. The specific question was addressed as to whether the intact structure of polyenic chain of vitamin A is needed for the substrate activity of retinylphosphate. It was found that the first reaction occurs even with the biologically inactive perhydromonoene retinylphosphate (pRP) to form mannosyl pRP. However, this compound was unable to carry out the second reaction. Studies on temperature dependence (Arrhenius plots) showed that the synthesis of mannosylretinylphosphate occurs with two slopes and a transition temperature, whereas the synthesis of mannosyl pRP (the inactive analog) occurred with one slope. This finding suggests that the double bonds of the vitamin may somehow be involved in the carrier function across the membrane. Lack of reversibility of reaction 1 in the absence of detergent and ready reversibility of this reaction with pRP further supports the idea that mannosylretinylphosphate is rendered cryptic to enzyme 1, once synthesized, whereas the pRP derivative is available (probably not mobile) within the membrane and thus inactive in membrane glycosylation.

We have found that cell surface receptors for epidermal growth factor (EGF) are greatly enhanced by retinoid treatment of cultured cells. Not the affinity but the number of surface receptors is greatly increased by the retinoid treatment by 5 to 10 fold. Receptors for insulin and for Concanavalin A were not affected to the same extent, even though a 1.2-fold increase for Con A receptors was noted. As the number of EGF-receptors increased, the cell division rate decreased and the synthesis of procollagen $_1$ and $_2$ was also increased.

The resulting picture suggests that vitamin A or retinoic acid induced changes in cell surface glycoproteins responsible for hormone reception. Antagonism between tumor promoter TPA and RA was found in these parameters.

HUMAN TISSUE STUDIES SECTION - (1) Conducts carcinogenesis studies on the pathogenesis of human cancer, especially carcinomas, using tissues from human target organs and from experimental animals, by organ culture and related methods; (2) conducts cellular and molecular investigations concerning both the function and differentiation on human epithelial cells and the interactions between carcinogens and these epithelial cells; (3) conducts experimental and clinical investigations to investigate the mechanism(s) of variation in carcinogenic susceptibility among people; and (4) investigates the problem of extrapolation of carcinogenesis data among species.

Model systems for human carcinogenesis: Carcinogenesis researchers have strived for decades to interpret data obtained from animal studies in terms of their applicability to humans. The carcinogenic hazard of environmental chemicals has been determined primarily from studies with experimental animals and from retrospective epidemiological investigations. An experimental approach to the direct study of carcinogenesis in human tissues and cells has been recently developed to assess the: a) mechanisms of carcinogenesis in human cells, b) variation of carcinogenic susceptibility among individuals, and c) validity of the extrapolation of carcinogenesis data from experimental animals to the human situation. Studies of carcinogenesis can now be conducted directly in human target tissues. In parallel with studies of human tissues, an appropriate animal model for each target site is being investigated. This approach provides a link between carcinogenesis studies using experimental animals and carcinogenesis in humans.

Model systems have been developed for human bronchus, colon, esophagus, peripheral lung, and pancreatic duct. These model systems have three major facets: 1) collection of viable human tissues; 2) in vitro maintenance of the tissues; and 3) xenotransplantation of human tissues into immunodeficient animals to assess tumorigenicity of suspected malignant cells transformed in vitro. Human tissues can be exposed to carcinogens and to anticarcinogens while in culture and/or as a xenograft. Explant cultures of human tissues have the advantage of retaining normal cellular differentiation and the 3-dimensional intercellular relationships. Monolayer epithelial cell cultures are being initiated from explants and used for carcinogenesis studies.

The long-term organ culture of human bronchus, colon, esophagus, peripheral lung and pancreatic duct was previously reported. During this fiscal year the serial passage and clonal growth of human bronchial and esophageal epithelial cells in serum-free medium and without feeder-cells provides new opportunities for the study of carcinogenesis and differentiation in these human cells.

The culture of target epithelial tissues and cells from experimental animals and humans in a controlled experimental setting has allowed a series of interlocking studies including: 1) metabolism of chemical carcinogens; 2) DNA damage by carcinogens; and 3) function, differentiation and transformation of human epithelial cells.

I. The Metabolism of Environmental Chemical Carcinogens

Studies of carcinogen metabolism were conducted in tissues from experimental animals and humans.

Cultured human bronchi, esophagus and colon were found to activate, metabolically, the following procarcinogens into forms that bind to cellular macromolecules including DNA: polynuclear aromatic hydrocarbons -- 7,12-dimethylbenz[a]anthracene (DMBA), 3-methylcholanthrene, benzo[a]pyrene (BP), and dibenz[a,h]anthracene; N-nitrosamines -- N-nitrosodimethylamine (DMN), N-nitrosodiethylamine (DEN); hydrazine -- 1,2-dimethylhydrazine (1,2-DMH); a mycotoxin -- aflatoxin B1 (AFB1); aromatic amines -- 2-acetylaminofluorene and a protein pyrolysis product, Trp-P-1.

Important differences in the metabolic activation of cyclic N-nitrosamines were found among human tissues. Human bronchus activated N-nitrosopiperidine (NPd), N-nitrosopyrrolidine (NPy), and N,N'-dinitrosopiperazine (DNP). Human colon activated NPy, but binding of NPd was not found. Neither NPy nor NPd were bound to DNA in cultured human esophagus.

The metabolic pathways leading to the formation of DNA adducts in cultured bronchus, colon and esophagus have been defined for BP, DMBA, AFB1 and DMN. The pathways to organic-extractable and water-soluble metabolites of BP were qualitatively similar in all 3 tissues; sulphate esters and glutathione conjugates were the major water-soluble metabolites.

The extrapolation of carcinogenesis data among animal species depends in part on qualitative and quantitative differences between metabolic activation and deactivation of procarcinogens. Therefore, the metabolism of BP by cultured tracheobronchial tissues from different species -- human, bovine, hamster, rat and mouse -- has been investigated. The total metabolism, as measured by both organic solvent-extractable and water-soluble metabolites of BP, was substantial in the respiratory tract from humans and from animal species susceptible to the carcinogenic action of BP. The ratio of organic-extractable metabolites to water-soluble metabolites was greater than one in hamster, human and C57Bl/-6N mouse, but less than one in rat, bovine and DBA/2N mouse, suggesting that determination of both activation and deactivation pathways are important in assessing carcinogenic risk of a chemical. Sulphate esters and glutathione conjugates were the major water-soluble metabolites in all animal species; tetrols and diols were the major organic extractable metabolites. The level of *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene, the proximate carcinogenic form of BP, was three times higher in C57Bl/6N, than in DBA/2N mouse trachea. *Trans*-9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene was the major metabolite formed by cultured hamster trachea. The binding levels of BP to cellular DNA were quite similar in all tissues, although slightly higher binding was observed in hamster trachea. Wide interindividual variation in the binding of BP to DNA was seen in tissues from outbred species. The major BP-DNA adducts in all animal species were formed by interaction of benzo[*a*]pyrene diol-epoxide with the 2-amino group of deoxyguanosine. Both stereoisomeric forms of (±)-(7 ,8)-dihydroxy-(9 ,10)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I) reacted with deoxyguanosine, the (7*R*)-form being the most reactive. No difference in the relative distribution of the various adducts was seen between the species, except in the CD rat where BPDE-deoxyadenosine adducts accounted for 20% of the total modification. In cultured hamster trachea the persistence of the different adducts was similar. In conclusion, the metabolism of BP is qualitatively similar in tracheobronchial tissues from both humans and animal species in which BP has been experimentally shown to be carcinogenic.

An individual's risk of developing cancer may depend, in part, on the balance between activation and deactivation of chemical procarcinogens. We have previously found wide quantitative differences (50- to 150-fold) in carcinogen bound to DNA in cultured human tissues. This wide interindividual variation is similar in magnitude to that found in pharmacogenetic studies of drugs. Whether or not these interindividual differences in carcinogen binding values reflect similar differences in oncogenic susceptibility to these procarcinogens, is an important question for investigation. For example, in a combined epidemiology-laboratory study, we are comparing the metabolism of BP in cultured non-tumorous bronchial mucosa from cancer and non-cancer patients. Patient history and clinical findings are obtained from interviews and hospital records. Lung cancers are classified by cytochemistry and both light and electron microscopy. Macroscopically normal bronchial explants, collected at time of surgery or immediate autopsy, are maintained in chemically defined medium for 7 days. BP (1.5 M) is then added for 24 hr. Binding levels of BP to mucosal DNA (pmoles per 10 mg DNA) are being measured as part of this study. In the initial 79 patients (21 without and 58 with lung cancer), the interindividual variation among samples from single cases is less than 2-fold. When compared to binding levels in non-cancer patients (18, mean; 2-82, range), higher levels are found in bronchi from patients with (a) epidermoid-differentiated carcinomas including combined epidermoid-adenocarcinomas with a well-differentiated epidermoid component (34; 2-111; $p < 0.01$) or (b) mucous-differentiated cancers with a non-glandular pattern (35; 11-96; $p < 0.025$), while no significant differences are found in bronchi from patients with glandular, mucous-differentiated cancers (15; 1-45). Patient age, sex and history of tobacco smoking and consumption of alcholic beverages are not confounding factors for the associations between binding levels and tumor types.

In summary, human bronchus, colon and esophagus can metabolize polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines, aromatic amines, and mycotoxins that are found in the environment and/or tobacco smoke. Metabolic pathways and carcinogen-DNA adducts were generally similar among animal species, including humans. Wide quantitative differences in carcinogen binding to DNA was found among different people. The relationship between this finding and an individual's oncogenic susceptibility to environmental procarcinogens requires further study. Finally, organ specificity of chemical procarcinogens may depend in part on inter-tissue differences in metabolism of the organotropic carcinogens. Quantitative and, in the case of cyclic N-nitrosamines, perhaps qualitative differences have been found among human tissues.

II. DNA Damage by Carcinogens

DNA damage is considered to be important for the oncogenic effects of many chemical carcinogens. As noted above, we have extensively studied the metabolism of chemical procarcinogens and have identified their DNA adducts in cultured human tissues and cells. The next logical step in these investigations is to determine the intra- and inter-genomic distribution of these adducts, DNA repair processes in human epithelial cells, and the biological consequences of these carcinogen-DNA interactions. Such studies are now feasible because of recent advances in molecular biology made by others and the following contributions by the HTSS and coworkers: (a) development of ultrasensitive enzymatic radioimmunoassay for the quantitative measurement of femtomole amounts of antigens including carcinogen-DNA adducts, (b) hybridoma production of monoclonal antibodies to carcinogen-DNA adducts, and (c) methods for the serial culture of human bronchial epithelial cells.

By combining radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), we have developed a very sensitive immunoassay -- ultrasensitive enzymatic radioimmunoassay (USERIA). Experiments were conducted to modify RIA or ELISA so that antigens or antibodies could be measured by the USERIA procedure, and to compare the sensitivity among these immunoassays. So far, USERIA has proved to be the most sensitive immunoassay. It is 100- to 1,000-fold more sensitive than RIA in the detection of rotavirus, cytomegalovirus, and cholera toxin. The procedure was modified to determine covalent binding of chemical carcinogens to DNA. The USERIA procedure which measures benzo[a]pyrene (BP)-DNA adducts and acetylaminofluorene (AAF)-DNA adducts was 100-fold more sensitive than RIA. As few as 3 fmole AAF-DNA adducts in 10 ng DNA can be measured by non-competitive USERIA and 2 fmole in 1 ng DNA by competitive procedures. With similar procedures, 4 fmole of BP-DNA adducts in 10 ng DNA can be detected by non-competitive USERIA, and 10 fmole in 100 g DNA (1 molecule of BP-DNA adduct per 107 nucleotides) by competitive methods.

Monoclonal antibodies to carcinogen-DNA should provide investigators with highly specific reagents for both clinical and molecular studies. We have initiated a program to produce such monoclonal antibodies. For example, mouse myeloma cells (P3x63) were fused with spleen cells from BALB/c mice immunized with AFB1-DNA adducts. Hybrid cells were grown in selective medium and tested for production of antibody-secreting hybridomas. Clones secreting monoclonal antibodies binding specifically to AFB1 modified DNA have been obtained and characterized. Monoclonal antibodies against other carcinogen-DNA products are being prepared.

Most studies of DNA repair in mammalian cells have been conducted in cultured fibroblasts and lymphocytic cells. With the successful culture of human bronchial cells, discussed in a later section, DNA repair is now being studied in normal human epithelial cells. Using alkaline elution methodology, developed by Kohn and coworkers, we have found that human bronchial epithelial and fibroblastic cells repair DNA damage by x-irradiation, metals, i.e., chromic compounds, DMBA or BPDE I. These studies are being extended to asbestos and to other chemical carcinogens and cocarcinogens.

III. Function, Differentiation and Transformation of Human Epithelial Cells

Defined methods to grow replicative cultures of normal human bronchial epithelial cells without serum or Swiss mouse 3T3 feeder-cells have been developed. These cells can be subcultured several times; and they will undergo 35 population doublings and have the expected epithelial cell characteristics of keratin, desmosomes and blood group antigens. Further, mitotically quiescent cells will differentiate into cells with beating cilia, a characteristic of normal bronchial epithelium. Dissociated single cells form colonies when plated at low density. In vitro carcinogenesis experiments with normal bronchial epithelial tissue and cell cultures have yielded populations of cells which have abnormal characteristics. These phenotypically altered cells (PACS), which have the keratin epithelial cell markers, have extended population doubling potentials, abnormal human karyologies and abnormal serum and growth factor requirements. The tumorigenic potential of these isolates are being assessed in athymic nude mice.

We are also studying the mechanism of asbestos cocarcinogenesis. Exposure to asbestos is known to cause a substantial increase in bronchogenic carcinoma in people who smoke tobacco. To measure toxicity, asbestos (UICC samples; 0.1 to 100 g/ml) was added to human bronchial epithelial cells that had been subcultured 24 hrs previously at clonal density (525 cells/cm2). When compared to glass fibers, asbestos caused a statistically significant ($p<0.05$) decrease in cell population doubling rate. Chrysotile was approximately 10-fold more cytotoxic than either amosite or crocidolite. A single exposure of amosite asbestos (100 - 1000 g/ml) caused focal epithelial hyperplasia and atypical squamous metaplasia in human tracheobronchial explants. Amosite fibers were shown by both scanning and high voltage transmission electron microscopy to penetrate cultured epithelial cells. Short fibers (<12) were found in the cytoplasm of the cells within 6 hrs, whereas longer fibers incompletely entered the cells. Other investigators have shown that human pulmonary alveolar macrophages react to asbestos by cell surface ruffling and coat the fibers with hemosiderin. Epithelial cells did not show increased cell surface activity, and only small membrane sleeves around non-coated fibers were observed at the points of asbestos penetration. *In vitro* investigations offer the potential to elucidate the acute and chronic effects of asbestos and other fibrous materials on human epithelial cells.

The serial cultivation of human esophageal epithelial cells under clonal growth conditions has also been achieved both under feeder cell and feeder-free conditions. Due to recent improvements in cultivation methods, the cells can be grown in the complete absence of serum. Experiments utilizing these cells in carcinogenesis studies are in progress. Cells exposed to Ni2SO4 gave rise to colonies with sustained growth properties. These cells are now being assessed for other properties indicative of cell transformation.

IV. Other Activities

This section has been responsible for training intramural and extramural investigators in the techniques for (a) culturing human epithelial tissues and and cells and (b) enzyme immunoassays to measure carcinogen-DNA adducts and oncofetal antigens. Members of our staff have co-organized both national and international scientific meetings, i.e., U.S.-Japan Collaborative Cancer Program Conference entitled "Interspecies Correlations in Chemical Carcinogenesis;" ICN-UCLA Symposium on Cellular and Molecular Biology, entitled "Mechanisms of Chemical Carcinogenesis;" and Tissue Culture Association meeting entitled "Epithelial Cell Culture." Members of our staff also serve on intramural and extramural committees, i.e., NCI Committee on Special Inquiries, Environmental Pathology Committee of the International Academy of Pathology, and Program Committee, III World Lung Cancer Congress.

Collaborative studies with the Department of Pathology, University of Nairobi, Kenya, and the Departments of Pathology and Immunology, Cancer Institute, Beijing, Peoples Republic of China, have been initiated. Our scientists have visited these institutions and biochemical epidemiology studies are in progress. Future exchanges of staff and materials are planned.

IN VITRO PATHOGENESIS SECTION: (1) Develops *in vitro* models for studying the pathogenesis of major forms of human cancer utilizing epithelium from both human and animal tissues; (2) utilizes these culture systems in biochemical, cytological and biological studies to investigate the mechanism of carcinogenesis and to develop approaches to the prevention of cancer; (3) studies the stages in the pathogenesis of cancer to identify markers for preneoplastic lesions so that such lesions can be arrested or reversed prior to their becoming a threat to life; (4) develops procedures for evaluating the biological potential of lesions induced by chemical or physical carcinogens in mammalian tissues *in vitro*.

This section has directed its efforts toward both developing *in vitro* model systems to study chemical carcinogenesis in epithelial cells and using these systems to study the mechanisms of tumor initiation and promotion. The classic model for induction of squamous cancer by chemicals, mouse epidermis, has been adapted for *in vitro* study. Previous investigations had demonstrated that this model is a close *in vitro* analogue of the mouse skin carcinogenesis system *in vivo*. *In vitro*, epidermal cells proliferate and differentiate, metabolize carcinogens, repair DNA damage, and respond to tumor promoters like epidermis *in vivo*.

Regulation of Epidermal Growth and Differentiation: Previous results from this laboratory have indicated that extracellular calcium concentration regulates epidermal proliferation and differentiation. Culture medium of 0.02 - 0.09 mM calcium concentration selects for proliferating cells which have morphological, immunological and biochemical characteristics of basal cells. Culture medium of > 0.1 mM induces epidermal differentiation resulting in cessation of proliferation, vertical stratification, cornification and sloughing of mature squames. The regulation of differentiation by calcium is not associated with changes in cyclic nucleotide levels but appears dependent on a functioning Na+-K+ ATPase pump as it is inhibited by ouabain and accelerated by high extracellular K+. The results suggest that epidermal differentiation is triggered by calcium but may depend on flux of other ions such as Na+ or K+ as the cellular mediator.

Quantitative Assay for Carcinogen-induced Altered Differentiation: The capability to selectively grow basal cells in low calcium medium and induce differentiation in high calcium has provided an assay to select for cells with altered differentiative responses. We have found that after carcinogen exposure in primary cultures of mouse keratinocytes, some cells resist the Ca++ signal to differentiate and continue to proliferate under high Ca++ conditions, producing countable foci which stain red with rhodamine B. The development of epidermal cell lines with high cloning efficiency has provided an opportunity to study this carcinogen-induced change in differentiation as a clonal assay to enhance quantitation of the frequency of this event. In both assays, the number of foci produced is directly dependent on carcinogen dose, and each focus appears to be clonal in origin. Cells derived from a number of foci have not been tumorigenic when first tested in syngeneic or nude mice, but become tumorigenic with prolonged passage producing squamous cell carcinomas. Studies utilizing skin from BALB/c mice initiated in vivo by administration of 25 ug dimethylbenz[a]anthracene (DMBA) suggested that resistance to induced differentiation was a preneoplastic characteristic. Sixty percent of dishes with epidermal cells from initiated skin contained foci resistant to calcium-induced differentiation, while epidermal cells from untreated mouse skin yielded only 2 foci in over 100 dishes. These findings suggest that resistance to induced differentiation is characteristic of cells from initiated skin and may be a fundamental change in initiation.

Phenotypic Markers for Malignant Epidermal Cells: The availability of normal, preneoplastic, and fully malignant keratinocytes in this laboratory has facilitated the study of specific markers associated with acquisition of malignant potential. Malignant keratinocytes retain many of the differentiative properties of normal epidermis, but resist calcium-induced differentiation. Thus, a small number of malignant cells can be selected from a large excess of normal cells by altering calcium levels, an analogy to the assay described above for carcinogen-treated normal cells. Malignant cells have high activity of gamma glutamyltranspeptidase, while this enzyme in normal cells is not measurable by histochemical procedures. Preneoplastic cell lines vary in detectable gamma glutamyltranspeptidase. The keratin intermediate filament cytoskeleton of malignant epidermal cells differs from normal cells in that it contains several new keratin peptides in the 50-60K region and many fewer desmosomes. Malignant cells, like normal cells, contain abundant pemphigoid antigen and secrete a factor which is a mitogen and comitogen for thymocytes.

Molecular Regulation of Epidermal Specific Differentiation Products: Our studies have indicated that initiation of carcinogenesis is associated with a change in normal differentiation. In order to understand this association at the molecular level, the regulation of specific differentiation products is being explored. Polyadenylated mRNA from epidermis was isolated and shown to contain the message for three major keratins (69K, 63K and 62K). Double-stranded reverse transcripts were prepared, tailed with dCMP and annealed with plasmid vector pBr322 previously cleaved with PsTl and tailed with dGMP. The recombinants containing a tetracycline resistance gene were used to transform E. coli strain RR1, and transformants were screened for keratin sequences by filter hydridization with 32P cDNAs of electrophoretically purified keratin in RNA. Of 480 transformants screened, approximately 30 were positive. Plasmid DNA has been prepared from several transformants and used to select complementary RNA from total skin poly(A)RNA for translation These cDNA recombinants will provide specific gene probes to analyze the functional changes in gene expression in epidermis associated with carcinogen and tumor promoter exposures and in preneoplastic and malignant cells where expression of keratins appears to be altered.

Determinants for Susceptibility to Carcinogenesis: Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases, specific genetic changes have been associated with increased risk, but in many examples, polygenic influences appear more likely. To date, biochemical epidemiological studies have focused only on genetic differences in carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact, it seems likely that factors associated with the expression of neoplastic change would play an important role in host susceptibility. The development of animal strains through selective breeding with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. This laboratory has utilized the SENCAR mouse strain for susceptibility studies since this strain is especially sensitive to chemically induced skin carcinogenesis. We have compared a variety of SENCAR properties to those of the low susceptibility BALB/c mouse. Increased susceptibility of SENCAR mice to skin carcinogenesis relative to BALB/c is not accounted for by differences in metabolism of the polycyclic aromatic hydrocarbon carcinogen DMBA or in binding of aromatic amine carcinogens to DNA. Sensitivity to carcinogenesis is a property of the SENCAR skin itself since skin grafts to the backs of athymic nude mice from SENCAR, but not BALB/c mice, developed tumors with high frequency after initiation and promotion. SENCAR, but not BALB/c, mice developed significant numbers of skin tumors with promoter alone in the absence of initiation suggesting that initiated cells may preexist constitutively in SENCAR mice. The preexistance of untreated cells in SENCAR skin was also suggested by the growth of calcium-resistant foci in cultures of SENCAR epidermal cells which were not exposed to carcinogens.

A variety of studies have been performed to determine a basis for the presence of initiated cells in SENCAR epidermis. Defective DNA repair capacity could not be demonstrated since the levels of host cell reactivation of UVL-irradiated Herpes simplex virus and direct activity of polymerase were identical in SENCAR and more resistant strains. SENCAR epidermal cells had 1.2 to 2.0 times the level of epidermal growth factor binding seen in BALB/c cells, but there was no quantitative correlation with sensitivity to carcinogenesis when CD-1 and AKR strains were included in the comparison. EGF binding in cells from all strains responded identically to exposures to phorbol esters and retinoids.

Epidermal cells of both SENCAR and BALB/c strains contain proviral DNA sequences for xenotropic type C RNA viruses, but spontaneous expression occurs more readily in BALB/c. A defect in immune surveillance was suggested by an 8-fold decrease in epidermal Langerhans cells, the skin macrophage, in SENCAR vs. BALB/c epidermis in culture, but confirmation of this deficiency in vivo is still pending. It is anticipated that future studies will provide insight into the molecular basis for genetically determined cancer susceptibility in SENCAR and suggest clues for further study of susceptibility determinants in humans.

Immunological Techniques to Study the Interaction of Carcinogens With DNA. The interaction of carcinogens with DNA has been studied by a unique methodology pioneered by this Section. Antibodies have been developed in rabbits against guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin-(8-yl)-2-aminofluorene (G-8-AF), the major guanosine adducts formed *in vivo* and *in vitro* by the interaction of nucleic acids with the aromatic amine carcinogen 2-acetylaminofluorene (AAF). Antisera have also been developed against DNA substituted with the 7,8-diol 9,10 epoxide of benzo[a]pyrene (BPDE) yielding the trans (7R)-benzo[a]pyrene-N2-deoxyguanosine (BPdG) as the major antigen. These antisera, which are highly specific for adducts, have been used to develop highly sensitive quantitative immunoassays for monitoring carcinogen binding and for morphological localization of binding sites. Using the G-8-AAF and G-8-AF antibodies, radioimmunoassay (RIA) studies indicate that the extent and pattern of AAF-DNA binding varies among cell types is determined at the cellular level and can be experimentally modified. In collaboration with Dr. Brian Laishes at the McArdle Laboratory for Cancer Research, RIA has been used to detect C-8 adducts in liver and kidney DNA of male Wistar-Furth rats fed 0.02% or 0.04% 2-AAF either continuously for up to 16 weeks or for a specific time followed by an interval for repair. With dietary AAF, substantial levels of binding (80 fmoles/ g DNA) were already observed in the liver DNA after 24 hours and with continuous feeding reached a plateau of approximately 230 fmoles/ g DNA at 30 days and thereafter. During the first week of continuous feeding, about 80% of the total C-8 adducts in the liver DNA were deacetylated (dG-8-AF) and the proportion of dG-8-AF increased to 97-100% by 15 and 30 days of feeding for the 0.04% and 0.02% 2-AAF diets respectively. In separate experiments, rats were fed 2-AAF for 3, 7 or 28 days, the carcinogen diet was discontinued and the liver adducts assayed at 1, 7 and 28 days after removal of the 2-AAF. When dietary 2-AAF administration was for 3 or 7 days, approximately 70% of the C-8 adducts remained on the liver DNA 7 days after a return to control diet, and 7-35% remained at 28 days. However, when dietary administration of 2-AAF was for 28 days, most of the C-8 adducts were still present after a 28 day repair interval. In all experiments, the formation of C-8 adducts in kidney DNA averaged 10-15% of the liver values and followed the same binding and repair profiles as liver. These results demonstrate that removal of specific adducts may be preferential during liver carcinogenesis and adducts may persist for a prolonged time period after withdrawal of carcinogen exposure.

Antisera to BP-DNA has a high affinity for native substituted DNA and has been particularly useful for morphological studies. Collaborative studies with Dr. David Kaufman at the University of North Carolina have utilized electron-microscopic immunohistochemical techniques to visualize BPDE-DNA adducts in DNA fibers. Over 60% of adducts can be visualized by these techniques. Cellular immunofluorescence studies have shown that virtually 100% of cells exposed to BPDE demonstrate bright nuclear fluorescence which is abolished by treatment with DNAase. RNAase abolished particularly intense staining associated with nucleoli. By 24 hours after BPDE exposure, immunofluorescence was no longer visualized, suggesting removal of adduct during the carcinogen-free interval to levels below detectability. The quantitative analysis of BPdG formation was enhanced by the development of ELISA and USERIA assays in collaboration with the Human Tissue Studies Section. These enzymatic amplification assays have yielded sensitivity in the range of less than 1 femtomole and allow detection of adduct from biological samples exposed to small quantities of

B(a)P. Using the ELISA assay, samples of human lung and white blood cells from individuals exposed to B(a)P through lifestyle (smoking) or occupation (coke oven or shale retort workers) are being monitored for adducts. The collection of the clinical material is being coordinated by Dr. I.B. Weinstein of Columbia University.

Mechanism of Action of Tumor Promoters and Antipromoters: Tumor promotion by phorbol esters has been an area of intense study in this Section. During the past year, we have begun to define a program of changes induced by the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse epidermal basal cells cultured under low calcium conditions. In collaboration with Dr. Michael Gottesman, the induction of specific protein synthesis was studied by two dimensional gel electrophoresis after pulse labeling with [35]methionine. While overall protein synthesis was moderately inhibited by TPA, the synthesis of 5 specific proteins was increased. Three of these proteins (25K, 55K and 70K) were either not synthesized or synthesized at low rates in untreated cells, while two proteins (35K and 50K) were also synthesized in controls but to a lesser extent than in TPA-treated cells. The synthesis of the 35K protein is reduced by the antipromoter fluocinolone acetonide and enhanced by retinoic acid. Maximum induction of all proteins was observed at 6 hours after TPA exposure. By 24 hours, synthesis rates of these proteins had returned to near basal levels even if TPA exposure was continued. Pulse-chase studies indicated that these proteins were not degradation products, which may have resulted from TPA exposure, and that the 25K and 35K protein appear to be rapidly turned over. [32PO4] labeling indicated that only the 55K protein was significantly phosphorylated and that TPA did not induce a qualitative change in the pattern of phosphorylation of epidermal proteins. The specific stimulation of these 5 proteins supports a model of tumor promoter action in which promoters induce a specific program of changes in macromolecular synthesis in the epidermis. Many aspects of tumor promotion by phorbol esters in skin have suggested that cell selection plays an important role in the process. We have provided evidence that the basis for cell selection resides in a heterogeneity in responsiveness of basal cells to phorbol esters. Our studies have demonstrated that basal cells respond to TPA exposure by the induction of the enzymes ornithine decarboxylase (ODC) and epidermal transglutaminase (ET), and by a stimulation of DNA synthesis. Studies with ODC induction of cultured basal cells exposed to TPA have revealed response kinetics which vary with TPA concentration, suggesting that two populations exist with differing sensitivities to TPA. Likewise, the induction of ET occurs in a subpopulation of basal cells which then terminally differentiate and slough from the culture dish. The remaining cells are then resistant to transglutaminase induction by increasing calcium concentration in the culture medium, a condition which elevates ET in controls. These cells are also resistant to the inhibition of DNA synthesis which occurs when medium calcium is elevated. Thus, TPA appears to have opposing effects on specific basal cell subpopulations. Since elevated ET activity is associated with epidermal differentiation, it appears that TPA is able to both induce and inhibit differentiation in subsets of basal cells. Cells which are resistant to the differentiation inducing effects of a single dose of TPA respond to a second TPA exposure with a stimulation of DNA synthesis but without an induction of ET. These results suggest that TPA selects for a population which responds with a proliferative stimulation and selects against a population which is induced to differentiate. This heterogeneous responsiveness in skin would cause redistribution of basal cell subpopulations, could clonally expand initiated cells and would be crucial to the promoting action of phorbol esters.

When human epidermal cells are exposed to TPA, induction of terminal differentiation predominates, leading to sloughing of many keratinocytes. Simultaneously, a cell type previously unseen in epidermis emerges to repopulate the culture dish. Thus, cell selection may be a primary action of TPA on tissues from several species.

PERINATAL CARCINOGENESIS SECTION - (1) Investigates the induction of cancer in experimental animals before birth and during infancy, with the goal of identifying factors responsible for the initiation and growth of tumors of childhood and infancy in man; (2) utilizes techniques of chemical synthesis, biochemistry, histopathology, immunology, and endocrinology to identify carcinogens to which both, individually and in combination, the fetus and neonate are particularly vulnerable, and to identify host factors which qualitatively modify their biological effects; and (3) develops measures for prevention of cancer in children or in later life as a response to conditions of high susceptibility to carcinogens during fetal life or childhood."

The program of this section continues to have two major components. A. Tumor induction experiments in animals. These studies seek to expand the limited data base now available from which to define the nature of prenatal risk of exposure to carcinogens and its relation to the major neoplastic diseases of man. Their purpose is to identify characteristic changes in susceptibility to chemical carcinogenesis as the stages of prenatal life progress from blastocyst to embryo to fetus, and after birth as neonates progress to juveniles and then mature to adults. Qualitatively, these studies seek to characterize (1) differences in the spectrum of organ systems affected by a given class of chemical carcinogens as development progresses; (2) how these spectra may vary from one species to another; (3) and, within a given organ system, how specific tumor types may arise depending on the stage of differentiation and development at which carcinogen exposure occurs. Quantitatively, dose/response relationships in the induction of tumors and related lesions are investigated for selected classes of chemical carcinogens during different stages of prenatal and postnatal development. These data contribute to the estimation of relative human risk during prenatal and postnatal life. Studies of this sort require contract-supported resources for the housing and maintenance of animals, for their treatment, and for collection and histologic processing of normal and diseased tissues. B. Studies on mechanisms related to prenatal susceptibility to carcinogenesis. In order to investigate and interpret the physiologic bases for susceptibility differences and to provide a rational basis for extrapolation to man, studies are in progress on several factors which appear to play major roles in determining age dependent, organ specific susceptibility to major classes of genotoxic chemical carcinogens. These include (1) the role of cellular proliferation, and variation in susceptibility to neoplastic transformation during specific stages of the cell cycle; (2) variations in capacity to repair damage to DNA in various organ systems and in different species during development; (3) the role of cocarcinogenic agents in promoting the phenotypic expression of neoplastic transformation, presumably consequent to carcinogen-initiated changes in the genome of target cells; (4) the relative roles of maternal and fetal metabolism in determining the organotropism and quantitative effects of chemical carcinogens of different classes in fetal and adult rodents; and, most uniquely relevant to transplacental carcinogenesis; (5) the role of state of differentiation and of its controlling factors, including both exogenous substances and cell-cell interactions, in controlling the expression of neoplastic transformation in selected organ systems, especially the kidney. In order to accomplish these studies, which cover a wide range of specialties and areas of expertise, it has been necessary to establish collaboration with several groups of investigators both within and outside NIH, including laboratories in foreign countries. Studies on the relation of specific stages of the cell cycle to carcinogenic susceptibility have been carried out in collaboration with colleagues at the University of North Carolina School of Medicine; investigations on transplacental carcinogenesis in primates, with the NINCDS and the University of California at Davis; and studies on the biology of neoplastic liver cells in rats and mice, with investigators at the American Health Foundation, Valhalla, New York.

Previous reports have documented indirect studies in this Section on the roles of maternal and fetal metabolism in determining fetal susceptibility to transplacental carcinogens in rodents and primates by carcinogenesis experiments involving agents selected from the following three classes of chemical carcinogens: agents which act directly without metabolic processing; metabolism-dependent agents with short-lived chemical intermediates including nitrosamines; and metabolism-dependent agents with long-lived metabolic intermediates, such as the polynuclear aromatic hydrocarbons.

Carcinogenesis studies in primates. During the past year, studies on a direct acting alkylating agent, ethylnitrosourea (ENU), have been continued in the Old World (African) monkey, *Erythrocebus patas*, and have been extended to an unrelated Asian species, the rhesus (*Macaca mulatta*). Previous results, suggesting that the earlier stages of prenatal development in primates are exceptionally susceptible to carcinogenesis have been confirmed by additional new data.

Transplacental carcinogenicity studies with ENU in the rhesus monkey have revealed that, like the patas, offspring of animals given ENU during the early stages of gestation developed lethal tumors, especially of connective tissues and including embryonal tumors of the lung and lymphoreticular neoplasia. All neoplasms detected so far have arisen during the first year of postnatal life. No tumors have been seen in rhesus monkeys exposed to comparable doses of the carcinogen as adults. This result confirms the significance of previous studies in the patas indicating the quantitatively greater susceptibility of primates to carcinogens during prenatal life, and demonstrates that results previously obtained in the patas are not unique to that species, but are applicable to other species of subhuman primates and, with high probability, must be considered predictive for man.

Organ culture studies on renal differentiation. Improvements have been made in the organ culture system previously developed for the *in vitro* differentiation of kidney rudiments from mice and rats, in order that renal differentiation and its relation to the expression of the neoplastic phenotype may be systematically investigated. It was shown that in this organ culture system both kidney rudiments from 15 day fetuses and primary undifferentiated renal tumors induced in newborn rats would grow for a period of 10 to 20 days and, in the case of kidney rudiments and at least one of the tumors, would differentiate. Primary undifferentiated blastemal cell renal neoplasms have now been established as serially transplantable tumors in the F344 rat, and have been shown to be adaptable to maintenance in organ culture also. Attempts are now under way to manipulate and induce the differentiation of such tumors, in order to understand the interspecies differences in morphology and patterns of growth of perinatally induced renal tumors, by comparing the undifferentiated tumors regularly inducible in the rat with the purely epithelial renal tumors of adult type induced in mice by similar treatments.

Cell cycle and susceptibility to carcinogenesis. The role of specific stages in the cell cycle, and of cell replication, in enhancing susceptibility to carcinogenesis has been studied by refining existing approaches to inducing cell synchrony during the regenerative hyperplasia which follows partial hepatectomy in the rat. When the proportion of hepatocytes in S was shifted in time by hydrocortisone administration, the period of increased vulnerability to hepatocarcinogenesis was shifted by a comparable interval. This confirms previous, rather fragmentary, data that had suggested that the S phase of DNA synthesis is a fraction of the cell cycle during which susceptibility to chemical carcinogenesis is increased. Methyl(acetoxymethyl)nitrosamine (DMN-OAc) was adopted as the carcinogen for continuation of these studies. DMN-OAc, studied in detail previously as a model compound, is a carrier form of the presumed reactive metabolite of dimethylnitrosamine. The cell cycle experiment was performed using DMN-OAc followed by prolonged dietary administration of phenobarbital as a promoter of liver tumor development. This protocol has resulted in the appearance of very high multiplicities of tumors in the rat liver after a single exposure to the carcinogen, and a virtually negligible incidence of tumors in other organ systems. This experiment is being concluded at the present time.

Role of gamma-glutamyl transpeptidase (GGT) in carcinogenesis. The mechanism of the association between high GGT levels and susceptibility to toxicity from azaserine in cultured cell lines has been further explored. Not all carcinogenic or toxic amino acids are related in the same fashion as azaserine to GGT content in target cells, and it now appears that simple functioning of the enzyme for transport of the carcinogen across cell membranes is not the entire answer to this complex question. In particular, neither ethionine nor the gamma-glutamyl derivatives of certain mushroom toxins, 1,1-disubstituted hydrazines, are comparable to azaserine in their patterns of toxicity in a battery of cell lines differing in GGT content. Alternative explanations for the role of GGT in organ specificity of azaserine toxicity and carcinogenicity are being tested.

DNA damage and repair in prenatal carcinogenesis. Far reaching conclusions on the role of DNA repair in determining susceptibility to prenatal carcinogenesis in various organ systems, especially the liver and brain in rats and mice, have been based, by others, on published observations that tumors in the rat liver are never induced by alkylating agents administered transplacentally. The demonstration reported above that phenobarbital is a potent promoter of the development of latent neoplastic liver cells into tumors suggests a method for further testing the hypothesis that the fetal rat liver, although it sustains damage from transplacental carcinogens, repairs that damage effectively and eliminates damage from hepatocyte genomes that would otherwise result in transformation to the neoplastic phenotype. Studies to test an alternative hypothesis have been designed and are now under way. Many transformed cells may in fact be induced transplacentally in the rat liver by exposure to alkylating agents, but in the absence of cocarcinogens administered postnatally, their probability of proliferating to generate tumors may be negligible. In the event that a high incidence of liver cell tumors is demonstrated in the livers of rats given transplacental ENU followed by postnatal phenobarbital, it will require a major reevaluation of current thinking on the role of repair processes as determinants of organ specific carcinogenesis.

Alkaline sucrose gradient studies were previously reported on excision repair of damaged DNA and the effects of alkylating agents in cells cultured from fetal livers and brains of rats and mice. These studies had demonstrated a significant lack of repair capacity in the fetal rat brain in comparison with the mouse brain or the liver of either species. More recent studies by the alkaline elution procedure utilizing tissues obtained directly from rodent fetuses confirmed the overall pattern observed in the previous studies. While these results are satisfying in view of the much greater susceptibility of the rat to carcinogenesis in the developing nervous system, they fail to explain why liver cell tumors are induced in the mouse in high multiplicity, while none are ever seen in response to comparable treatment in the rat. The studies with phenobarbital now under way may resolve this problem. Meanwhile, experiments are continuing in order to establish the relative efficiency of excision repair in different organ systems in both rats and mice at different stages of development, following prenatal exposure to alkylating agents and to other toxic and carcinogenic compounds.

Application of research in perinatal carcinogenesis. The relevance of perinatal carcinogenesis to the programs of national and international organizations, especially in the fields of disease prevention and safety evaluation, has continued to result in frequent requests for participation in formal meetings organized to discuss the utilization of research findings in this field by regulatory agencies and others concerned with the evaluation and application of toxicologic investigations. During the past year, such activities have included participation in the New York Academy of Sciences meeting on possible occupational risk of brain tumors in the chemical industry; advice to the Consumer Product Safety Commission on the significance of carcinogenicity studies on the fire retardant for infants' sleepware, tris (2,3-dibromopropyl) phosphate; and activity as co-organizer and co-editor of the proceedings, jointly with the Environmental Protection Agency, of the Symposium on Organ and Species Specificity in Chemical Carcinogenesis, Raleigh, N.C., March 1981.

In May of 1981 this Section was transferred to the Frederick Cancer Research Center, Fort Detrick, to participate in formation of the new Laboratory of Comparative Carcinogenesis.

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Modulation of Cell Surface and Growth Parameters by Retinoids

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PI:	Luigi M. De Luca	Research Scientist	LEP	NCI
Other:	Anton M. Jetten	Expert	LEP	NCI
	Louis D. Dion	Staff Fellow	LEP	NCI
	Carol Silverman-Jones	Microbiologist	LEP	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Differential Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Retinoic acid (RA) enhanced the binding of ^{125}I-labeled epidermal growth factor (EGF) to various fibroblastic and epidermal cell lines by increasing the number of receptor sites available for binding. This effect is specific for EGF-receptors and is not the result of increased internalization and/or degradation of EGF. Rate of restoration of EGF-receptor binding after "down regulation" by excess EGF is faster in RA-treated cells, suggesting that RA stimulates the synthesis of this cell surface receptor. At the same time RA decreases growth rates of a variety of cell lines. Soft agar colony formation by 3T3 A31-1-BP-2 cells is decreased by RA and increased by the tumor promoter TPA. RA and retinol prevented colony formation in the presence or absence of TPA. Exceptionally, the fibroblastic rat kidney cell line NRK 536-3 (SA6) responded to RA by a remarkable increase in colony forming ability in soft agar medium, but only when given to the cells in the presence of sarcoma growth factor. RA by itself had no activity. RA increased, whereas TPA decreased, the biosynthesis of secreted procollagen in 3T6 fibroblasts and in 3T3 A31-1-BP-2.

PHS-6040
(Rev. 2-81)

Objectives: To study the molecular mechanisms by which retinoids increase the adhesive properties of spontaneously transformed mouse fibroblasts in culture; in particular to characterize cell surface glycoproteins which may be responsible for the vitamin A-induced enhanced adhesion, and to understand mechanisms of growth control.

Methods Employed:

Cell Culture: Mouse fibroblast cells BALB/c 3T6 and 3T3 A31-1-BP-2 were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and supplemented with 100 /ml each of penicillin and kanamycin and 100 μg/ml of streptomycin. Normal rat kidney fibroblast cells NRK 536-3 (SA-6) were grown in the same medium except that calf serum was substituted for fetal calf serum.

Binding of Epidermal Growth Factor: Cells were grown in complete medium in 35 mm cluster dishes. At the time of the EGF binding assay, medium was removed and cells washed twice with 2 ml binding buffer (DMEM containing 1 mg/ml of BSA and 50 mM Bes pH 6.0). Then 1 ml of binding buffer containing 0.825 ng of ^{125}I labeled mouse EGF (Collaborative Research, 1.4 x 10^5 dpm) was added to each well. After 60 min at 37°C, unbound ^{125}I-EGF was removed, cells were washed four times with 2 ml buffer and solubilized in 0.75 ml lysing buffer (0.1 M Tris-NCl, pH 7.4 containing 0.5% SDS and 1 mM EDTA) and the wells washed twice with 0.5ml lysing buffer. Radioactivity was counted in a Packard γ-counter. Nonspecific binding was determined in the presence of 10 μg unlabeled EGF.

Internalized radioactivity was determined by washing the cells three times with ice-cold binding medium after incubation with ^{125}I-EGF, followed by treatment with 1 ml solution of acetic acid (0.2 M pH 2.5) containing 0.5 M NaCl for 6 min at 4°C. Cells were then rinsed two times with 0.5 ml of the same solution and then solubilized in lysing buffer. Cell-surface associated ^{125}I-EGF was calculated from the difference between total cell-associated and internalized radioactivity.

[^{3}H]-Thymidine Incorporation. Cells were grown in DMEM containing 10% serum until confluent. Cells were then incubated in DMEM containing 0.25% serum for an additional three days. [^{3}H]-Thymidine incorporation was determined by incubating the cells for 24 hrs in DMEM containing ^{3}H-thymidine (2.5 μM, 0.2 Ci/mmol). After incubation, cells were washed and then solubilized in 200 1 lysis buffer and added to 3 ml cold 10% TCA. After 15 min in ice, radioactivity was collected on Millipore filters.

Anchorage Independent Growth. Trypsinized cells were added to complete medium supplemented with 0.3% agar (Difco) and aliquots of 2 ml were pipetted onto a base layer of 3 ml of complete medium plus 0.5% agar in 60 mm petri dishes (Falcon). Both base and upper layers contained the additions as indicated. Plates were generally incubated for 2 weeks. At the last day of incubation 0.5 ml of p-iodonitro tetrazolium violet (0.5 mg/ml) was added and incubation continued for another 24 hrs. The number of colonies were assayed with the aid of a Omnicon image analysis system (Bausch and Lomb).

Labeling of Secreted Proteins. Cells were grown in complete medium in the presence or absence of 10^{-6} M retinoic acid for 72 hrs. In some instances TPA (100 ng/ml) was added at 48 hr and incubation continued for another 24 hrs. At that time, incorporation of ^{35}S-methionine into secreted products was determined. Cells were washed twice with medium without serum and methionine and then incubated in the same medium containing 20 μCi/ml of ^{35}S-methionine (990 Ci/mmol). After a 2 hr incubation period, medium was removed and cells were centrifuged at 1500 x g for 10 min. Then a 100% TCA solution was added to the supernatent until a final concentration of 10% was reached. After the addition of 50 μg of BSA as a carrier, medium was incubated in ice for 30 min and then centrifuged for 30 min at 20,000 x g. Precipitate was washed with ether and solubilized in 10 mM Tris-NCl buffer (pH 7.4) containing 0.5% SDS.

In the case of 2-^{3}H-mannose and ^{14}C-proline incorporation, the procedure was the same as for ^{35}S-methionine, except that medium containing low glucose or medium without proline was used, respectively.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed according to the method of Laemnli. Polyacrylamide gradient electrophoresis was performed using 6-22% acrylamide. Gels were processed and prepared for fluorography as described by Bonner and Laskey.

Major Findings:

Action of Retinoids on EGF Binding. Retinoic acid enhances the binding of ^{125}I-labeled EGF to various fibroblastic and epidermal cell lines. It has no marked effect on the affinity of this growth factor for its receptor, but it increases the number of EGF receptor sites. Retinoic acid has little effect on the binding of Concanavalin A and insulin, indicating the specific nature of the action of retinoids on some cell surface glycoproteins. Treatment of cells with the phorbol ester TPA and retinoic acid shows poor antagonism between these compounds on EGF binding. Retinoic acid treatment does not seem to have a significant effect on the rate of internalization and degradation of EGF. At 0°, internalization is strongly inhibited in both retinoic acid-treated and control cells. When exposed to high concentrations of EGF, both retinoic acid-treated and control cells "down-regulate" their EGF receptors. And, although the growth-rate of retinoic acid-treated cells is about half that of control cells, the rate at which EGF binding capacity is restored after down-regulation is about three times as fast as in control cells. The simplest explanation for this result is that the synthesis of EGF receptors occurs at a faster rate in retinoic acid treated cells.

Action of Retinoids on Anchorage Independent Growth. One in vitro property closely associated with the transformed state in vivo is the ability of cells to grow progressively while suspended in semi-solid medium. Retinoids prevent the formation of colonies in soft agar in 3T3 A31-1-BP-2 cells. In this cell line TPA increased colony formation by almost 7-fold, while PDA, a biologically inactive phorbol ester, did not enhance colony formation significantly. Both retinol and retinoic acid, in the presence or absence of TPA, prevented colony formation in soft agar. The pyrimidyl analog of retinoic acid was inactive. The behavior of retinoids on NRK 536-3 (SA6) contrasts that on 3T3 A31-BP-2 cells. In this rat kidney cell line, retinoids stimulate dramatically the colony forming ability induced by sarcoma growth factor. The phorbol ester TPA is less effective in inducing anchorage independent growth than SGF; simultaneous treatment of these cells with TPA and retinoic acid enhances colony formation in soft agar significantly. Retinoids by themselves do not induce anchorage independent growth of these cells, suggesting that this group of compounds do not act like growth promoters, but enhance the effects of growth promoters in this system. Although the stimulation of growth in soft agar by various retinoids correlates well with the specificity with which they bind to the cytosolic binding proteins, no binding proteins were detectable in the cytosol of these NRK cells.

Effect of Retinoic Acid on Secreted Proteins. Treatment of mouse fibroblast 3T6 cells with retinoic acid causes a dramatic change in the electrophoretic profiles of secreted proteins labeled by either ^{35}S-methionine, 2-^{3}H-mannose or ^{14}C-proline. Three bands are especially affected: two bands with molecular weights of 155,000 and 135,000 daltons are increased while a band of 35,000 daltons is decreased upon retinoic acid treatment. These bands are also labeled by 2-^{3}H-mannose indicating that they are glycoproteins. The 155 and 135K dalton bands comigrate with pro $\alpha 1$ and pro $\alpha 2$ and are sensitive to collagenase digestion. Upon pepsin treatment, these bands are converted to 110 and 90K daltons bands. When the mannose-labeled glycoproteins are subjected to pepsin digestion, the 110 and 90K dalton bands do not appear since collagen $\alpha 1$ and $\alpha 2$ do not contain mannose. The results strongly suggest that these two bands are the pro $\alpha 1$ and pro $\alpha 2$ chains of pro-collagen. Treatment of 3T6 cells with TPA reduces the labeling of these bands; simultaneous treatment with TPA and retinoic acid also causes a marked reduction in these bands. These results correlate well with our previous findings that in 3T6 cells retinoic acid cannot prevent the action of TPA. In 3T3 A31-BP-2 cells, the effect of retinoic acid and TPA on secreted proteins is different from 3T6 cells. Retinoic acid itself has no major effect on secreted products while TPA causes a reduction in labeled procollagen. In 3T3-A31-BP-2 cells, retinoic acid prevents this reduction induced by TPA.

The 35,000 dalton band has been identified as the major excreted protein (mep).

Significance to Biomedical Research and the Program of the Institute. Alteration in cell surface adhesion and, in general, in recognition phenomena appears to be a result of neoplastic transformation. Therefore, agents which modify cell surface characteristics to resemble the normal phenotype are of obvious interest in attempts to characterize cell surface traits responsible for invasion, growth in soft agar and other peculiarities of transformed cells.

It has been found that, in addition to their function as chemopreventive agents in chemical carcinogenesis, retinoids can alter cell surface morphology and adhesion of cultured spontaneously transformed mouse fibroblasts, BALB/c 3T12-3 cells, to resemble the "normal phenotype". At the molecular level, it appears that retinoids modify adhesive properties of transformed cells by their action on the biosynthesis of specific cell surface glycoproteins. Retinoids also seem to affect profoundly the availability of receptors for growth factors, such as EGF-receptors on the cell surface.

Thus, it is reasonable to expect that this project will contribute to our understanding of the basis for the decreased adhesion and greater invasive potential of neoplastically transformed cells and that it will allow a better understanding of mechanisms of growth control.

Proposed Course.

The following seem to be logical developments of this research: characterization of cell surface glycoproteins responsible for altered growth and adhesion of neoplastically transformed cells; modulation of the synthesis of these glycoproteins by retinoids and antagonism by tumor promoters such as phorbol esters. Study of the mechanism responsible for the increase in EGF receptor sites caused by retinoids.

Publications:

Sasak, W., De Luca, L. M., Dion, L. D., and Silverman-Jones, C. S. Effect of Retinoic acid on cell surface of glycopeptides of cultured spontaneously-transformed mouse fibroblasts/Balb/c 3T12 cells). Cancer Res. 40: 1944-1949, 1980.

Jetten, A. M. Action of retinoids and phorbol esters on cell growth and the binding of epidermal growth factor. Ann. N.Y. acad. Sci. 359, 200-217, 1981.

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Dion, L. D., De Luca, L. M., Colburn, N. H. Phorbol ester-induced anchorage independence and its antagonism by retinoic acid correlates with altered expression of specific glycoproteins. Carcinogenesis, 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04488-05 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Carcinogenesis Studies in Cultured Colon from Experimental Animals and Humans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herman Autrup	Senior Staff Fellow	LEP	NCI
Other:	John James	Guest Worker	LEP	NCI
	Roland Grafstrom	Visiting Fellow	LEP	NCI

COOPERATING UNITS (if any)
University of Maryland, School of Medicine, Baltimore, MD; National Naval Medical Center, Bethesda, MD

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 1	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A model system for studying chemical carcinogenesis in colon has been developed. Human colon can generally be maintained 14 days and rat colon at least 63 days as explant cultures. Cultured human and rat colon have the ability to enzymatically convert various classes of chemical carcinogens, such as polycyclic aromatic hydrocarbons, N-nitrosamines, mycotoxins, protein pyrolysis products and hydrazines, into metabolites which react with cellular macromolecules, such as DNA and protein. A wide variation among people in the binding of both benzo(a)pyrene (BP) and 1,2-dimethylhydrazine (DMH) was seen. The data showed a skewed distribution that strongly suggested a distribution more complex than uni-modality. A positive correlation in the binding of BP to DNA was seen between colon and duodenum from the same individual, the binding level being highest in the duodenum. The carcinogen-DNA adducts for aflatoxin B_1 (AFB), BP, N-nitrosodimethylamine and DMH have been identified and the persistence of the AFB-DNA modification have been studied in human colon. The effects of various suspected inhibitors and enhancers of colon carcinogenesis have been investigated on the metabolism of BP and DMH.

Objectives: To develop a model system for studying carcinogenesis in human colon. To compare the metabolism and the effect of chemical carcinogens in human colon to that in experimental animals. To study the effect of various co- and anti-carcinogens on the metabolism of these carcinogens.

Methods Employed: Explant cultures of colon; autoradiography, xenotransplantation; electron microscopy; high resolution light microscopy; isolation of cellular macromolecules; high pressure liqud chromatography; thin layer chromatography.

Major Findings: Human colonic epithelium has been cultured as explants in a chemically defined medium for periods up to 24 days. The viability of the explants was shown by the preservation of the ultrastructural features of the colonic epithelial cells and by active incorporation of radioactive precursors into cellular DNA and protein. Colonic tumor tissues were successfully maintained as explant cultures using conditions identical to normal tissue. The explants were generally maintained up to 14 days under these conditions. Adult rat colon was maintained with a modified structural integrity for up to 63 days under similar conditions.

Metabolism of various carcinogens in cultured human colon has been investigated. Non-tumorous colonic tissue was collected at the time of either "immediate autopsy" or surgery from patients with or without colonic cancer (221 cases). After 24 hrs in culture, explants were exposed to radioactive-labeled carcinogen for another 24 hrs and the binding to cellular DNA was measured by radiometric methods.

The binding levels of BP to human DNA showed a skewed distribution that strongly suggests more than uni-modality. The mean binding level was 6.3 pmoles/10 mg DNA (median 4.3; range 0.2-39.3). There was no significant difference in binding level between surgical tissue obtained from colon cancer patients (mean 7.1; median 3.5), surgical tissues from non-colon cancer patients (6.4; 4.1) or from immediate autopsies (7.3; 5.4). The major adduct was formed by trans-addition of BP diol epoxide I to the exocyclic 2-amino group of guanine. The binding of BP to DNA was inhibited by addition of either butylhydroxyanisal at selenium -- both at non-toxic doses -- to the media. No qualitative changes were observed in the distribution of organo-soluble metabolites. A positive correlation between the binding of BP and DMH to DNA was found ($r = 0.500$; $p < 0.001$; 127 cases), while no correlation was found between the binding of BP and DMN ($r = 0.56$; $p > 0.05$; 15 cases). The binding value of DMH to DNA also showed a wide variation among people, the distribution being similar to that of BP binding to DNA. Slightly higher mean binding levels were seen in tissue from immediate specimens. No significant age difference was observed, while females (age group 51-70; sigmoid colon) have a higher binding level than men. When the anatomical segments were compared, the highest binding of DMH was observed in the sigmoid and transverse colon and the lowest in rectum and the descending colon. A higher binding level was found in duodenum than in transverse colon from the same patient.

AFB, a colonic and hepatic carcinogen in rats, was metabolized by cultured human colon. (The mean binding level of AFB to DNA (1.8 ± 2.0) was lower than that of BP (5.9 ± 5.7) in the same patient. No correlation was found between the two sets of binding levels ($r = 0.136$; $p > 0.05$; 24 cases). One major AFB-DNA adduct was formed by addition of AFB-2,3-oxide to the 7-position of guanine. This reaction product is unstable and the imidiazole ring will open to stabilize the molecule. This ring opened form was the major adduct when human colon was cultured for up to 3 days after AFB treatment for 1 day.

The conjugation of 2-naphthol was investigated in normal and tumor tissue. Sulphate conjugation was the major pathway in normal tissue, while glucuronidation was the predominant pathway in tumor tissue.

The metabolism of BP, DMN, DMH and AFB has been studied in both colonic and duodenal tissue from the same patient. The level of biotransformation was significantly higher for all four carcinogens in the duodenum. The metabolite pattern of BP and the BP-DNA adducts were similar in the two tissues, and a positive correlation in the binding level ($r = 0.82$; $p < 0.001$; 15 cases) was observed.

Microsomal fractions were isolated from human duodenum and colon. The microsomal fractions showed minimal contamination of mitochondria as measured by the absence of succinate-cytochrome c reductase activity. Cytochrome P-450 was recorded in microsomes from both colon and duodenum. Furthermore, NADPH-cytochrome c reductase activity was detected. The microsomes also diethylated 7-ethoxycoumorin and metabolized BP to phenols, quinones and dihydrodiols.

The relationship between the genetic control of DMH-carcinogenesis and the enzymatic activation of DMH has been studied in a mouse model. L57Bl/Ha, a non-susceptible, and ICR/Ha, a susceptible species to DMH-induced colon carcinogenesis, were treated with [^{14}C]-DMH with or without prior treatment with DMH. The level of 7-MeGua and 0^6-MeGua -- analyzed by HPLC -- was determined in four different fragments of the colon. The highest level of modification was seen in the distal colon and a positive correlation between level of alkylation and number of tumors or "microtumor" index in ICR/Ha and C57Bl/Ha, respectively. No difference in the ratio of 0^6-MeGua and 7-MeGua was seen between the two species. Pretreatment with DMH did not have any significant effect on the alkylation pattern.

Significance to Biomedical Research and the Program of the Institute: A colon explant system has been developed which can be used for carcinogenesis studies. Chemical carcinogens which are metabolically activated and form carcinogen-DNA adducts by human colon may induce cancer in this tissue in humans. Determination of binding levels of carcinogens in a greater group could identify people at high risk for chemical induction of colonic cancer. The model system provides a link between experimental animal studies and the human situation, as comparative studies of animal and human tissues can be performed under similar controlled conditions. Human tissues obtained by immediate autopsy also allow a comparative study in various organs from the same individual.

Proposed Course: To continue improving the culture conditions for human colon. The effect of exposure of the explants to carcinogens and potential promotors of colon carcinogenesis will be investigated both biochemically and morphologically, using combinations of explant cultures and xerotransplantation.

The metabolism of N-nitrosopyrrolidine and protein pyrolysis products will be investigated in more detail. The study on the relationship between the genetic control of DMH-carcinogenesis and the enzymatic activation of DMH will be continued.

Publications:

Autrup, H., Harris, C. C., Schwartz, R. D., Trump, B. F., Smith, L.: Metabolism of 1,2-dimethylhydrazine by cultured human colon. Carcinogenesis 1: 375-380, 1980.

Autrup, H. Use of explant cultures to study the metabolism of polycyclic aromatic hydrocarbons in the gastrointestinal tract. In Mozsik, G., Hanninen, O., and Javor, T. (Eds.): Gastrointestinal Defense Mechanisms. Adv. Physical Sci. 1981. 29: 385-404, Budapest Akademiai Kiado and Pergamon Press, 1981.

Autrup, H., Jeffrey, A. M., and Harris, C. C.: Metabolism of benzo(a)pyrene by cultured human tissues. In Bjorseth, A., and Dennis, A. J. (Eds.): Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects. Columbus, Battelle Press, 1980, pp 89-105.

Autrup, H., Schwartz, R. D., Essigmann, J. M., Smith, L., Trump, B. F., and Harris, C. C.: Metabolism of aflatoxin B_1, benzo[a]pyrene and 1,2-dimethylhydrazine by cultured rat and human colon. Teratogenesis, Mutagenesis and Carcinogenesis 1: 3-13, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04490-05 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Evaluation and Prevention of Carcinogenic Effects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Umberto Saffiotti, M.D., Chief	LEP NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary objectives of this work are to examine current knowledge in the field of carcinogenesis and related fields and to identify criteria for the evaluation of carcinogenic effects of chemical and physical agents, for the evaluation of risk assessment methods, and for the prevention of cancer hazards in the human population.

Laboratory methods and biological models for the detection of carcinogenic activity of chemicals are examined and evaluated, with particular emphasis on animal models of carcinogenesis and in vitro models for carcinogenesis studies.

Criteria for the evaluation of carcinogenic risks from environmental and occupational human exposures are developed. Research approaches are outlined for studies in carcinogenesis mechanism addressed to the problems of occupational and environmental cancer prevention.

Objectives: To examine current knowledge in the field of carcinogenesis and related fields, to identify criteria for the evaluation of carcinogenic hazards in the human population and for assessment of their risk, and to plan research approaches, relevant to the study of occupational and environmental cancer prevention, for development in laboratory work.

Methods Employed: Examination and evaluation of biological models, methods and findings used for the detection of carcinogenic activity and the identification of mechanisms of carcinogenesis. Analysis of results of carcinogenesis studies, including experimental design, metabolism, pathology and statistical evaluations. Analysis of occupational and environmental exposure data and of interactions of carcinogens with human tissues. Review and documentation of environmental carcinogenesis data and evaluations. Organization of and participation in conferences and workshops. Participation in advisory groups for national and international organizations.

Major Findings: A theoretical model was developed to account for multiple concurrent causative factors in the etiology of cancer in human populations; this model accounts for the following factors: genetic and nutritional factors in all cancers; exposure to carcinogens through diet and through inhalation, and exposure to radiations, each in all cancers; exposure to occupational factors in 20-40% of cancers; to various other factors in 10-70% of cancers, and to unknown factors in all cases. The composite sum adds up to 600-700%. Further fractionation can be considered. Even small increments in any one factor may become determinants of cancer development if the other factors are approaching a permissive set of conditions.

Other contributions were made in the following activities:

1) Collaboration with Federal Agencies.

a) For the Occupational Safety and Health Administration (OSHA): review of a "Candidate List" of compounds considered for possible regulation as occupational carcinogens, as chairman of the NCI Ad Hoc Working Group.

b) For the U. S. Environmental Protection Agency (EPA): 1) review and advice on the EPA Genetic Toxicology Program through membership in its Assessment Panel; 2) advice on the development of the NCI-EPA collaborative research program.

c) For the National Institute of Occupational Safety and Health (NIOSH); collaboration in the development and review of the NCI-NIOSH collaborative research program, particularly in planning a conference on Occupational Carcinogenesis.

d) For the Federal Panel on Formaldehyde (Consumer Product Safety Commission): review and evaluation on the carcinogenic and toxic effects of formaldehyde.

2) International activities: (a) Invited lecturer, Symposium on Occupational Cancer, Helsinki, Finland, April 1981; (b) Invited lecturer, NATO Advanced Studies Institute, course on the use of human cells for the assessment of

risk from physical and chemical agents, San Miniato, Italy, August-September 1981; (c) Invited lecturer, XX International Congress on Occupational Health, Cairo, September 1981; (d) Chairman, Committee on Occupational Carcinogenesis, Permanent Commission and International Association for Occupational Health; (e) Participant in joint meetings, U.S.-Italy Cancer Program, and visits to Italian laboratories.

3) Other professional activities include: (a) service as President (1978-1982) of the Society for Occupational and Environmental Health; (b) Editorial activity as Associate Editor of "Teratogenesis, Carcinogenesis, Mutagenesis"; (c) invited speaker, Gordon Research Conference on Experimental Cancer Therapy, Plymouth, NH, July 1980.

Significance to Biomedical Research and the Program of the Institute: The national policies on environmental health and cancer prevention need to be based on sound scientific grounds. A solid and well-documented basis of research data and evaluations is necessary in the development of criteria for sound health protection policy. The experience obtained by analyzing and coordinating different methodological approaches to the study of the carcinogenic process -- at the human, animal, cellular and molecular level -- provides a strong basis for identifying specific criteria and priorities for public health and for further research. The evolution of scientific knowledge will necessitate a process of continuous evaluation of these criteria.

Proposed Course: Continuation of these activities, with emphasis on research approaches.

Publications:

Bogovski, P., Fishbein, L., Holmberg, B., Infante, P., Montesano, R., Ramel, C., Saffiotti, U. and Sorsa, M.: Panel discussion: Extrapolation from animal experiments to humans. J. Toxicol. Environ. Health 6: 1327-1335, 1980.

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Saffiotti, U.: The problem of extrapolating from observed carcinogenic effects to estimates of risk for exposed populations. J. Toxicol. Environ. Health 6: 1309-1326, 1980.

Saffiotti, U.: Training and research opportunities for Italian investigators in the U.S.A.: NIH perspectives. Proc. Symp. on Italian Biomedical Scientists in the United States: Selection, Research and Reentry. (In press).

Saffiotti, U. and Cortesi, E.: Carcinogenicity of pesticides: Review and evaluation of recent results. In Kimbrough, R. and Kahn, E. (Eds.): Pesticides and Human Health. Society for Occupational and Environmental Health, Washington, D.C., 1980. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04491-05 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Quantitative Studies on Concurrent Exposures to Numerous Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Umberto Saffiotti	Chief	LEP NCI
Other:	Jerry M. Rice	Chief, Perinatal Carcinogenesis Section	LEP NCI
	Winston D. Edwards	Visiting Associate	LEP NCI
	Enrico Cortesi	Visiting Fellow	LEP NCI
	Paul J. Donovan	Chemist	LEP NCI
	Takeo Kakunaga	Chief, Cell Genetics Section	LMC NCI

COOPERATING UNITS (if any)

Cell Genetics Section, Laboratory of Molecular Carcinogenesis, DCCP, NCI

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.9	0.6

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SUMMARY OF WORK (200 words or less - underline keywords)

Experimental studies are conducted on concurrent exposure of appropriate biological targets to numerous carcinogens of different classes, each administered at doses which would be expected to produce low or undetectable effects in the chosen system. Quantitative exposure/response studies are addressed to test the hypothesis that many different carcinogens can act synergistically to induce a significant level of carcinogenic response when given concurrently at individually subeffective doses. Mechanisms of action are studied to determine the most effective conditions of synergism. The biological models selected for these studies include in vitro assays for mutagenesis and neoplastic cell transformation and whole animal models. Analysis of dose-response relationships in carcinogenesis is conducted on data collected in a comprehensive literature survey, and on dose-response results obtained experimentally in the selected biological systems.

PHS-6040
(Rev. 2-81)

Objectives: To determine whether a marked carcinogenic effect can result from concurrent exposure to numerous different carcinogens, each administered at doses which would be expected to produce low or undetectable effects in the chosen target system; and to analyze the mechanisms of such interactions of effects and dose-response relationships in carcinogenesis.

Methods Employed: Biological model systems include the Salmonella mutation assay (Ames) and neoplastic transformation of Balb/c 3T3 mouse fibroblasts in culture (Kakunaga). Quantitative exposure protocols were used for graded doses of individual carcinogens and their combinations (up to 8 compounds together so far). The results were analyzed quantitatively. A new laboratory facility is being set up in Building 560 of the NCI Frederick Cancer Research Center. The new space was designed for research in tissue culture and for experimental animal pathology studies. Equipment and supplies were selected for the new facility.

I. Salmonella mutagenesis: Strains TA 98 and TA 100, responding respectively to frame-shift and to base substitution mutagens, were used with or without S-9 mix prepared from Aroclor 1254-induced rat liver.

II. Neoplastic transformation: The Balb/c 3T3 clone A31 mouse fibroblast cell line system was used. Several subclones were obtained from T. Kakunaga, Laboratory of Molecular Carcinogenesis, DCCP, NCI. Most studies were performed on clone A31-1-1, of which several subclones were studied to define their growth and transformation characteristics.

The cell system was set up following the original protocol (Kakunaga, Intl. J. Cancer 12: 463-473, 1973) with minor modifications. Culture conditions and use of media (MEM with 10% fetal bovine serum) and solvents were optimized. Each study included toxicity determinations (8 days) and neoplastic transformation experiments (5 weeks). Scoring of transformation results was based on the number of Type III foci, but the induction of Type II foci was also recorded. Twenty-four hours after plating 10^4 cells per 60mm Petri dish, chemicals under study were added to the medium and removed 72 hours later; medium was subsequently changed twice per week. Metabolic activators were not added.

Major Findings: I. Salmonella mutagenesis. In the previous two years, mutual inhibition was demonstrated for a mixture of 5 polycyclic aromatic hydrocarbons as well as for each individual combination of components; mutual synergism was demonstrated for a group of compounds belonging to different chemical classes: benzo[a]pyrene (BP), aflatoxin B_1 (AFB), benzidine (BZ) and safrole (SF), and their individual combinations, while ethylene thiourea was inactive. Synergism occurred at low levels of exposure where toxicity is minimal for all compounds. Another class of compounds, the aromatic amines, was studied because of a) data on carcinogenic effects in humans and in animals, b) the synergistic effects of benzidine mentioned above and c) the recent findings of high carcinogenic activity for benzidine dyes and their metabolic breakdown to several free aromatic amines. Analysis of the results confirmed the following findings in TA 98 and TA 100 for benzidine (BZ), 3,3'-dichlorobenzidine (DCB), 4-aminobiphenyl (4-AB), 2-naphthylamine (2-NA), 1-naphthylamine (1-NA), o-dianisidine (OD), o-tolidine (OT), and aniline (AN). The first four of these compounds are well known carcinogens, while the latter four compounds have low or negative activity. Dose-response mutagenicity data were obtained for all compounds except o-tolidine and aniline, which were not found to be mutagenic. BZ, 4-AB, 2-NA and 1-NA required metabolic activation by S9, but DCB and OD were mutagenic even without S9, although S9 greatly enhanced their effect. DCB was more active in TA 98, while 4-AB and 2-NA were more active in TA 100. Combination tests were conducted at equimolar doses (0.125 M/plate) with S9. Various permutations of combinations were studied, including mixture of the 8 compounds (resulting in inhibition in both strains), mixture of the 3 most active compounds, DCB, 4-AB and 2-NA (inhibition in TA 100 and additive effect in TA 98) and permutations of 2 compounds at a time: most of these showed approximately additive effects, a few were inhibitory (4-AB + OD and 4-AB + OT, in both strains), while the combination BZ+4-AB resulted in synergism in both strains. Tests of the benzidine dyes were conducted with S9 and riboflavin additions: direct black 38 was found highly mutagenic in TA 98 by the plate assay (15 times higher than background) and 10 times more active than that when tested by liquid incubation. Direct brown 95 and direct blue 6 were not active in any of these tests. It was noted with interest that direct black 38 was found to yield both BZ and 4-AB by metabolic breakdown; synergism of metabolically released free bases is suggested as a possible factor in the high carcinogenic effect of the dye. On a molar basis, direct black 38 was found to be about 5 times more active in plate assays than the amount of benzidine contained in the dye molecule.

II. Neoplastic Transformation in Balb/c 3T3 clone A31 cells. These studies, initiated in the Spring of 1979, have been continued and the methods further refined. Dose-response relationships for cytotoxicity and transformation were determined for the following carcinogens: the direct-acting N-methyl-N'-nitro-N-nitrosoguanidine, (MNNG), as well as BP, 3-methylcholanthrene (MCA), AFB and BZ which require different type of metabolic activation. The relative transforming activity on a molar basis was found to be in following order: BP>AFB>MCA and MNNG>BZ. Good quantitative reproducibility was obtained in several replicate tests. Studies on combined effects in this transformation system are under way. Results of toxicity tests generally indicate an additive pattern of cytotoxic responses for the combined exposures so far studied; transformation experiments so far also suggest additive effects.

III. Studies on cell density in the expression of transformation. A new study was undertaken to determine the dose-response effects of carcinogen exposures in the expression of transformation obtained after replating exposed cells at different cell densities. Kennedy and Little (1980) reported that cultures of the C3H 10T-1/2 mouse fibroblast cell line, exposed to a transforming dose of X-radiation, grown to near confluence and then replated at widely different densities, show a nearly constant rate of transformed foci per plate. These studies suggest that nearly all carcinogen exposed cells behave as potentially transformed cells but that the expression of transformation is inversely proportional to the number of cells replated. A possible explanation is that cells inhibit the expression of transformation in other cells. This behavior is being studied in Balb/c 3T3 clone A31-1-1 cells: the role of carcinogen-exposed and unexposed cells on the expression of transformation of carcinogen exposed cells is investigated under a range of carcinogen doses. The preliminary results are under analysis.

Significance to Biomedical Research and the Program of the Institute: Multiple concurrent exposures to many different carcinogens represent the actual conditions under which the human population is exposed to carcinogens. From prenatal life through childhood and adult life people are exposed to a large number, probably in the hundreds, of environmental carcinogens from different routes. Such "realistic" conditions of exposure have never been reproduced experimentally so far. The definition of the effect of this background low level exposure to individual carcinogens, acting together with the exposure to many others, is expected to provide information on the mechanisms of carcinogenesis in tissues exposed to multiple hits by different chemicals and on the role of multiple synergisms in carcinogenesis. The role of high-level exposures to single carcinogens, when superimposed on different kinds of multiple background exposures will also be studied. Wide-ranging implications can be projected for the understanding of basic chemical biological interactions in carcinogenesis, for mathematical models for dose/response extrapolation and for the evaluation of carcinogenic hazards in public health policies.

Proposed Course of Project: These projects were partly interrupted because of the relocation of the Laboratory to Frederick. It is proposed to continue and extend these studies in biological systems for mutagenesis, cell transformation and in vivo carcinogenesis, using additional types of carcinogens; mechanism studies will be further developed to elucidate the bases of interactions and their relation to the human response.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04504-09 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.H. Yuspa	Chief, In Vitro Pathogenesis Section	LEP	NCI
Other:	H. Hennings	Senior Chemist	LEP	NCI
	M. Poirier	Research Chemist	LEP	NCI
	M. Kulesz-Martin	Staff Fellow	LEP	NCI
	D. Roop	Expert	LEP	NCI
	J. Strickland	Research Chemist	LEP	NCI

COOPERATING UNITS (if any)
Dr. Ulrike Lichti,
Frederick Cancer Research Center
Determatology Branch, DCBD, NCI
Laboratory of Molecular Biology, DCB, NCI
University of Washington, Seattle, WA
Dr. Robert Goldman, Northwestern
University, Chicago, IL

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
In Vitro Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.0	3.5	4.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse and human epidermal cell cultures and epidermal cell lines are utilized as models for studying mechanisms of epithelial carcinogenesis in vitro. Low extracellular Ca^{++} concentrations in culture medium select for basal cells, while elevated Ca_{++} induces terminal differentiation apparently by modulating the flux of other cations. Carcinogen exposure in vitro or to mouse skin prior to culture results in the development of cell colonies which are clonal in origin and are resistant to Ca^{++} induced terminal differentiation. The basis for altered differentiation in skin carcinogenesis is being explored through gene cloning, and cDNA probes for the 62K and 69K keratins have been prepared. Studies to define the role of tumor promoters in skin carcinogenesis have revealed that phorbol esters act specifically on basal cells and induce a specific program of new protein synthesis. Phorbol esters affect subpopulations of basal cells in a heterogeneous way, inducing differentiation in one class and stimulating proliferation in another.

Objectives: To study cellular and molecular changes during stages of chemical carcinogenesis through the use of unique *in vitro* model systems designed to simulate well-studied *in vivo* models. Studies are directed to give insight into general changes occurring in mammalian cells during malignant transformation and specific molecular events which may be causative to the transformation process. Specific markers of the transformed phenotype are also being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed: This laboratory has developed and utilized mouse epidermal cell culture as a major model to approach the stated objectives. Previous studies have shown that this model functions biologically in a highly analogous fashion to mouse skin *in vivo*. Human epidermal cells obtained from neonatal foreskins have also been adapted to growth *in vitro* in recent years. *In vivo* studies utilizing the two-stage mouse skin carcinogenesis model and grafts of human or mouse skin into nude mice are also employed. A number of laboratory techniques are required to pursue the objectives. Morphology is followed by light and electron microscopy and histochemical staining. Macromolecular synthesis and growth kinetics are studied by biochemical and autoradiographic procedures. Cellular functions, including the production of specific differentiation products, are monitored by enzyme assays, gel electrophoresis, amino acid analysis and radioimmunoassay. The progression to the malignant phenotype is monitored by growth rates, soft agar assay, karyotypic abnormalities, enzymatic changes and injection of cells into nude or newborn mice. A number of immunologic techniques including cell surface antibody production, fluorescent staining, immunoprecipitation and radioimmunoassay are being performed to recognize the normal or altered phenotype. Isolation of specific mammalian genes is performed through the preparation of epidermal mRNA, reverse transcription and cloning of transcripts in plasmid pBr322. Cloned genes are characterized by filter hybridization and translation arrest assays.

Major Findings: The pursuit of this project has led to major new findings in three pertinent areas: 1) enhancement of model systems including improved culture methodology, transformation assay, and the identification and selection of preneoplastic cells; 2) new information concerning the biochemical and biological consequences resulting from carcinogen exposure; 3) increased understanding of the process of preneoplastic progression and the mechanism of tumor promotion and anti-promotion. In addition, confirmation of *in vitro* results by *in vivo* experimentation has been obtained.

During the past year, both our understanding of the fundamental biology of the mouse epidermal culture system and the usefulness of this model for carcinogenesis studies have been greatly enhanced. The recent discovery that ionic calcium is a critical regulator of epidermal growth and differentiation have provided a method to control and study each of these phases of the keratinocyte life cycle. At low ionic calcium concentrations in culture medium (0.02 - 0.09 mM), epidermal cells maintain a monolayer growth pattern with a high proliferation rate. Essentially 100% of the attached cells are in the proliferating cell pool. Differentiation continues but differentiated cells are discarded into the culture medium. Proliferating keratinocytes synthesize prekeratins and display the basal cell pattern of differentiation proteins by polyacrylamide gel electrophoresis.

When cells maintained under low calcium growth conditions are switched to medium with calcium content above 0.1 mM (standard commercial culture media are 1.2 - 1.8 mM), differentiation is induced. A variety of morphological, biochemical and immunological techniques have indicated that low calcium culture selects for basal cells while higher calcium levels induce differentiation in a manner closely resembling the in vivo process.

The mechanism by which calcium induces epidermal differentiation has been studied in some detail. We have determined that the Ca^{++} effect cannot be reproduced by many other cations nor can it be blocked by inhibitors of macromolecular synthesis, microtubule disrupters or several calcium antagonists. The Ca^{++} effect is independent of cyclic nucleotides, markedly accelerated by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and inhibited by the Ca^{++} ionophore A23187 and the inhibitor of Na-K ATPase, ouabain. Ouabain is ineffective as an inhibitor if extracellular K^+ is maintained at a high level. Furthermore, differentiation is accelerated in high extracellular K^+ and delayed in the absence of extracellular K^+. These results suggest a role for K^+ in epidermal differentiation.

The capability to selectively grow basal cells in low calcium medium and induce differentiation in high calcium has provided an assay to select for cells with altered differentiative responses. We have found that after carcinogen exposure in vitro some epidermal cells resist the Ca^{++} signal to differentiate and continue to proliferate under high Ca^{++} conditions, producing countable foci which stain red with rhodamine B. The number of foci produced is directly dependent on carcinogen dose. Each focus appears to be clonal in origin since flow cytometry has shown that the target population of primary epidermal cells has a bimodal DNA content (a mixture of diploid and tetraploid cells), while each carcinogen-induced focus is unimodal (either diploid or tetraploid). Cells derived from a number of foci have not been tumorigenic when first tested in syngeneic or nude mice. When these cells are maintained in culture for 6 months, with monthly subpassage, they become tumorigenic, producing squamous cell carcinomas. This suggested that resistance to induced differentiation was a preneoplastic characteristic. Support for this idea was provided by studies utilizing skin from BALB/c mice initiated in vivo by administration of 25 μg dimethylbenz[a]anthracene (DMBA). When epidermal cells from initiated skin were cultured in low Ca^{++} medium and then switched to high Ca^{++}, approximately 60% of the dishes contained resistant foci which stained with rhodamine. In contrast, epidermal cells from untreated mouse skin yielded only 2 focus containing dishes over 100 cultures. These findings suggest that resistance to induced differentiation is characteristic of cells from initiated skin and may be a fundamental change in initiation.

To improve quantitation of carcinogen induced alterations in terminal differentiation, methods have been developed for the clonal growth of mouse epidermal cells. Using fibroblast conditioned low calcium medium, primary cultures can grow at clonal density with an efficiency of up to 1%. Selected subclones (BK lines) have been obtained with cloning efficiencies of 5-10%. When BK-1 cells plated at clonal density are exposed to carcinogens, rhodamine + colonies resistant to calcium induced differentiation occur at a frequency of 1 colony per 100 cells exposed to a concentration causing 50% cytotoxicity. Cells from such colonies are currently being tested for tumorigenicity and dose-response characteristics of the assay are being studied. Concurrently, BK-1 cells have been used to establish a mutagenesis model for ouabain resistant foci. This assay requires an expression time of 6-7 days and a selective ouabain dose of 1 mM. Dose-response and mutation frequency experiments are in progress. The availability of normal, preneoplastic, and fully malignant keratinocytes in this laboratory has facilitated the study of specific markers associated with acquisition of malignant potential. In previous studies, we have shown that malignant keratinocytes retain many of the differentiative properties of normal epidermis. When malignant keratinocytes are grown in low calcium medium and then switched to high calcium, they resist the differentiation signal. Thus, a small number of malignant cells can be selected from a large excess of normal cells by altering calcium levels, an analogy to the assay described above for carcinogen-treated normal cells. Other markers are being explored. Malignant cells have high activity of gamma glutamyltranspeptidase, while this enzyme, in normal cells is not measurable by histochemical procedures. Preneoplastic cell lines vary in detectable gamma glutamyltranspeptidase. The keratin intermediate filament cytoskeleton of malignant epidermal cells differs from normal in that it contains several new keratin peptides in the 50-60K region and many fewer desmosomes. Preneoplastic cells have not yet been studied. Malignant cells contain abundant pemphigoid antigen, and this antigen has been isolated by immuno-precipitation and characterized as a 220K protein with disulfide linkages. This is the first definitive characterization of this antigen. Malignant epidermal cells also secrete a factor which is a mitogen and co-mitogen for thymocytes. Preliminary studies suggest that a similar factor is secreted by normal keratinocytes. Significant progress has been made toward the isolation and cloning of the genes for the major proteins synthesized by the epidermis, the keratins. Polyadenylated RNA (Poly(A)RNA) was isolated from epidermal homogenates and shown to contain keratin messages in a reticulocyte translation system. The translation of keratins was confirmed by immunoprecipitation products with keratin-specific antiserum. The keratins are the most abundant species of mRNA in the poly(A) fractions, and poly(A)RNA obtained from intact epidermis codes for 3 (69K, 63K, 62K) major keratins, while poly A RNA obtained from cultured epidermal cells is greatly reduced in the 69K and 62K messages but codes for the 63K keratin in abundance. Poly(A)RNA from skin was used as a template for reverse transcriptase and a double-stranded cDNA was prepared and tailed with dCMP. The tailed cDNA was annealed with plasmid vector pBR322 previously cleaved with PsTI and tailed with dGMP and these recombinants were used to transform E coli K strain RRI. Transformants were selected by tetracycline resistance, replica plated in microtiter plates and screened for keratin sequences by filter hybridization with [^{32}P] cDNA's of electrophoretically purified keratin mRNA. Of 480 transformants screened, approximately 30 were positive. Plasmid DNA has been prepared from several transformants and used to select complementary RNA from total skin poly(A)RNA for translation. Recombinant plasmids containing cDNA corresponding to the 69K and 62K keratin mRNAs have been identified. These cDNA recombinants will provide specific gene probes to analyze the functional changes in gene expression in epidermis associated with carcinogen and tumor promoter exposures and in preneoplastic and malignant cells where expression of keratins appears to be altered.

The epidermal culture system has been very useful for defining normal epidermal biology and for identifying alterations associated with carcinogen treatment. It has also provided valuable markers associated with neoplastic change in that tissue and promises to be useful for defining the molecular alterations in expression of these markers. In addition, the model has been used extensively to define specific and relevant changes produced by tumor promoters. During the past year, we have begun to define a program of changes induced by phorbol esters in mouse epidermal basal cells cultured under low calcium conditions. In collaboration with Dr. Michael Gottesman, the induction of specific protein synthesis was studied in TPA-treated and control basal cell cultures by two dimensional gel electrophoresis after pulse labeling with [^{35}S]methionine. While overall protein synthesis was moderately inhibited by TPA, the synthesis of 5 specific proteins was increased. Three of these proteins (25K, 55K and 70K) were either not synthesized or synthesized at low rates in untreated cells while two proteins (35K and 50K) were also synthesized in controls but to a lesser extent than in TPA treated cells. Increased synthesis of the 50K protein could be seen as early as 1 hour after TPA exposure, while maximum induction of all proteins was observed at 6 hours. By 24 hours, synthesis rates of these proteins had returned to near basal levels even if TPA exposure was continued. The amino acid analogue canavanine (at levels which inhibited protein synthesis to the same extent as TPA) or non-promoting analogues of TPA did not induce these proteins. Pulse-chase studies indicated that these proteins were not degradation products which may have resulted from TPA exposure and that the 25K and 35K protein appear to be rapidly turned over. [^{32}PO$_4$] labeling indicated that only the 55K protein was significantly phosphorylated and that TPA did not induce a qualitative change in the pattern of phosphorylation of epidermal proteins. Definitive identification of these proteins has not been made, but the specific stimulation of their synthesis supports a model of tumor promoter action in which promoters induce a specific program of changes in macromolecular synthesis in the epidermis. The 35K protein has been studied in detail since it has been identified as MEP (Major Excreted Protein), a secreted glycoprotein synthesized in large amounts by transformed cells. We have shown that MEP synthesis is also induced by TPA exposure in mouse skin *in vivo*. *In vitro*, after TPA, MEP is essentially the only protein macromolecule secreted by epidermal cells. The synthesis of both constitutive and TPA-induced MEP is suppressed by the antipromoter steroid, fluocinolone acetonide. Retinoic acid in concert with TPA enhances MEP synthesis. Unfortunately, the function of MEP and its possible role in tumor promotion are unknown at this time.

Many aspects of tumor promotion in skin have suggested that cell selection plays an important role in the process. Cell selection in a tissue whose structure is highly restricted in size requires selection both for and against cell types. This suggested that there could be heterogeneity in responsiveness of basal cells to phorbol esters. Basal cells respond to TPA exposure by the induction of the enzymes ornithine decarboxylase (ODC) and epidermal transglutaminase (ET), and by a stimulation of DNA synthesis. These markers were utilized to search for heterogeneous responses to basal cells. When the induction of the enzyme ornithine decarboxylase (ODC) is studied, the basal cell response kinetics are dose dependent. At low TPA concentration (1 ng/ml), ODC activity peaks at 3 hours. At higher concentrations (100 ng/ml), the 3 hour activity is depressed and peak activity occurs at 9 hours. This is consistent with the existence of two subpopulations with differing sensitivities to TPA. When the induction of the enzyme epidermal transglutaminase (ET) is studied, a similar result is seen. ET activity is increased 3-5 fold in basal cell cultures within 12-14 hours of TPA exposure. This is associated with morphological changes in about 50% of the cells at risk. These cells become round, cornify and slough from the culture dish within 24-48 hours, the time when ET activity returns to basal values. The remaining cells are then resistant to transglutaminase induction by increasing calcium concentration in the culture medium, a condition which elevates ET in controls. These cells are also resistant to the inhibition of DNA synthesis which occurs when medium calcium is elevated. Thus, TPA appears to have opposing effects on specific basal cell subpopulations. Since elevated ET activity is associated with epidermal differentiation, it appears that TPA is able to both induce and inhibit differentiation in subsets of basal cells. Cells which are resistant to the differentiation inducing effects of a single dose of TPA respond to a second exposure with a stimulation of DNA synthesis but without an induction of ET. These results suggest that TPA selects for a population which responds with a proliferative stimulation and selects against a population which is induced to differentiate. This heterogeneous responsiveness in skin would cause redistribution of basal cell subpopulation, could clonally expand initiated cells and would be crucial to the promoting action of phorbol esters.

Using a recently developed model for cultivation of human epidermal cells, studies were conducted to determine the effects of TPA on human cells for comparison with known effects on mouse keratinocytes. TPA did not stimulate DNA synthesis. TPA enhanced vertical stratification. Many cells sloughed by the third day and quantitative assays revealed that 50% of the population was composed of cornified cells as opposed to 8% in controls. A new cell type (TT cell) emerged at this time, which was triangular in shape, did not stratify, appeared to proliferate rapidly and became the predominant cell type within 1-2 weeks. TT cells formed few cornified envelopes, grew in the absence of TPA and formed colonies at low cell input. TPA enhanced TT colony formation 3-4 fold and decreased the doubling time of TT cells. Studies were performed to determine the origin of TT cells. TPA treatment of dermal fibroblasts did not yield TT cells. By immunofluorescence, TT cells lacked the epidermal antigens keratin, pemphigus or pemphigoid; tonofilaments and desmosomes were not seen by electron microscopy. Absence of staining with Factor VIII antibody and the lack of both melanosomes and DOPA oxidase staining indicated TT cells were not of endothelial or melanocyte origin. While TT cells, as well as dermal fibroblasts were positive for vimentin, by immunofluorescence, TT cells (unlike fibroblasts) were negative for leucine amino peptidase. Tests for Langerhans cells, including rosette formation and ATPase activity, were negative. These results suggest that the primary effect of TPA on cultured human epidermis is to induce terminal differentiation in keratinocyte population and to stimulate growth of a minor, as yet unidentified, cell type. Thus, cell selection may be a primary action of TPA on tissues from several species.

Significance to Biomedical Research and the Program of the Institute: The majority of human cancers are associated with environmental exposures and most of the tumors are of epithelial origin. Animal models have been extremely useful for bioassay and some mechanistic studies, but they are not as useful for investigations at the cellular level because of complicated interactions between host and environment as well as physiological variations within an experimental protocol. The use of cell culture systems, particularly those of epithelial origin, offers the opportunity to extend the present conceptual models of carcinogenesis mechanisms to a more basic and cellular level. In addition, cell cultures ultimately should be as useful and inexpensive as bioassay screening procedures to detect carcinogens and cocarcinogens in the environment. Many of our present basic concepts concerning the pathogenesis of cancer were developed from studies utilizing carcinogen painting on mouse skin. The irreversibility of initiation, the phenomena of cocarcinogenesis and tumor promotion, the role of hyperplasia and metabolism are examples of such concepts. The development of a cell culture system for epidermal carcinogenesis has been a major advance toward extending our knowledge of mechanisms of carcinogenesis. Earlier efforts were directed to proving that epidermal cells in culture responded to carcinogens and promoting agents as _in vivo_; in almost every parameter studied, this was the case. Differentiation, metabolism, proliferation, metabolic activation and covalent binding of carcinogens, and promoter responses were highly analogous to the _in vivo_ situation. These findings enhanced the validity of any subsequent observations made _in vitro_. For the last several years the model system has been utilized to a much greater degree to ask questions about mechanisms of transformation, carcinogen and promoter interactions, and the role of anticarcinogenic agents such as retinoids and steroids. The routine isolation of cells resistant to selection for terminal differentiation, as well as the development of nontumorigenic and tumorigenic cell strains, offers the opportunity to study the cancer phenotype and tumor progression. The role of differentiation in carcinogenesis and the mechanism by which promoting agents induce progression can also be studied. Antigenic markers have become useful tools to detect normal and abnormal states and to provide a rapid assay for differentiation. Greater understanding of mechanisms of tumor promotion and the stages of tumor progression provides an opportunity to devise schemes for intervention in the process of carcinogenesis prior to the development of overt malignancy. Evidence for such a possibility is already apparent from studies of steroid hormones, retinoids and local anesthetics. Finally, while bridging the gap between relevant animal models and _in vitro_ systems, this laboratory is simultaneously developing the analogous human tissue model _in vitro_. Thus, ultimately a chain of systems will be available to determine the relevance of findings in any one model for the entire spectrum of models including the human.

Proposed Course: This project represents an integrated comprehensive approach to understanding the biological changes associated with initiation and promotion of carcinogenesis and their underlying molecular mechanisms. Future studies are a logical extension of each component of the overall approach. In order to understand the regulation of normal epidermal differentiation, the calcium-modulated culture model will be explored in depth. The role of ions will be determined by direct measurement of intracellular ionic changes by flame photometry. Assays for determination of Na-K ATPase activity will be developed to further define the role of that enzyme in differentiation. In depth evaluations of the formation of cell-cell contact, desmosome formation, and communication through gap junctions will be performed. The results obtained with normal keratinocytes will be compared to those with preneoplastic and neoplastic keratinocytes. Additional markers will be studied in the 3 cell types. In particular, a detailed analysis of the cytoskeleton will be performed in collaboration with Dr. Robert Goldman at Northwestern University which will include morphological, immunological and biochemical studies.

Transformation studies utilizing resistance to induced differentiation will be expanded. Additional chemicals of varying initiating activity will be tested. Modification of the target cells at the time of carcinogen exposure will be utilized to attempt to enhance or inhibit the transforming event. Modifiers will be chosen which are known to alter initiation in mouse skin _in vivo_. Modifiers which can alter the extent or pattern of carcinogen binding to DNA will also be utilized to determine the effect on initiation. Immunological assays developed in this laboratory (see project Z01-CM-05177-01-LEP) will be used to monitor binding. Progression from the initiated cell to the malignant cell will be systematically studied to elucidate the temporal sequence of this change and to examine the capability of additional carcinogen or promoter treatment to accelerate progression. The development of clonal transformation assays will continue with the testing of other BK lines and dose-response studies. Parallel development of mutagenesis assays will continue and frequency comparisons for initiation and mutation under the same treatment conditions will be performed. As the murine epidermal system becomes routinely used for mechanistic questions, a parallel assay will be developed using human keratinocytes.

The isolation and characterization of specific expressed epidermal genes, in combination with the sensitive immunological probes for carcinogen binding, could allow the study of the functional and structural consequences of exposure to initiators and promoters. These probes will be used to monitor changes in the number of gene copies, gene location and regulation of expression. This approach will be extended to compare normal, preneoplastic and neoplastic cell types. Epidermal messenger RNA changes will also be monitored during transformation and after single exposures to initiators and promoters.

The discovery of the heterogeneity in responsiveness of basal cells to phorbol ester tumor promoters provides a theoretical basis for the cell selection apparent in promotion. This will be explored in great detail in the next year. Subpopulations will be isolated after promoter treatment and studied individually for specific markers. The number and character of TPA receptors will be analyzed on each population and their response to various pharmacologic agents will be explored. Antibodies to each cell type will be prepared in rabbits, and after absorption of anti-mouse activity, localization of each cell type in the epidermis will be studied by indirect immunofluorescence. The proteins induced by TPA will be studied in greater detail to see if any are directly involved in producing the biological responses seen. This will be studied in whole basal cell populations and in selected subpopulations. Reconstruction experiments with normal and initiated cells will assess the ability of phorbol esters to select initiated cells from a mixed population. Similar studies will be performed with agents such as mezerein and teleocidin. New _in vitro_ findings will be tested _in vivo_ on mouse skin to assure validity of the data.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04513-06 LEP

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Metabolism of Chemical Carcinogens and DNA Damage in Cultured Human Tissues and Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herman Autrup	Senior Staff Fellow	LEP	NCI
	Curtis C. Harris	Associate Chief, LEP and Chief, HTSS		
Other:	R. Grafstrom	Visiting Fellow	LEP	NCI
	J. Groopman	Staff Fellow	LEP	NCI

COOPERATING UNITS (if any)

University of Maryland, School of Medicine, Baltimore, MD; Massachusetts Institute of Technology, Cambridge, MA; Institute for Cancer Research, New York, NY; Cancer Institute, Beijing, Peoples Republic of China

LAB/BRANCH

Laboratory of Experimental Pathology (LEP)

SECTION

Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human bronchus, pancreatic duct and esophagus can be cultured for several weeks in a chemically defined medium. This controlled experimental setting provides an excellent in vitro system to study the metabolism of chemical carcinogens, including those found in tobacco smoke and the environment. Several classes of chemical carcinogens, polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines, aromatic amines and mycotoxins can be metabolically activated by human tissues. Epithelial cell cultures initiated from human bronchus and esophagus metabolized benzo[a]pyrene (BP), aflatoxin B$_1$ (AFB) and N-nitrosodimethylamine (DMN) into species which reacted with cellular DNA. The metabolic pathways leading to the formation of DNA adducts in explant and epithelial cell cultures have been defined by BP, 7,12-dimethylbenz[a]anthracene, AFB, and DMN. The pathways to organic-extractable and water-soluble metabolites of BP were qualitatively similar in all 3 tissues. The adducts between these carcinogens and DNA in human bronchus and esophagus are essentially the same as those found in experimental animals in which the chemicals are carcinogenic. Inter-individual differences in carcinogen-DNA binding values vary 50- to 150-fold.

Objectives: To determine the metabolic pathways of chemical carcinogens in target tissues of experimental animals and humans. To measure interindividual and inter-tissue variations in the metabolism of carcinogens.

Methods Employed: Explant culture and epithelial cell cultures of human and animal tissues; quantitative high-resolution light microscopic autoradiography; isolation of cellular macromolecules; high pressure liquid chromatography; enzyme assays.

Major Findings: Cultured human bronchial mucosa can enzymatically activate procarcinogens [polynuclear aromatic hydrocarbons: 7,12-dimethylbenz[a]anthracene (DMBA), 3-methylcholanthrene (MCA), benzo[a]pyrene (BP), and dibenz[a,h]anthracene (DBA); N-nitrosamines: N-nitrosodimethylamine (DMN), N-nitrosodiethylamine (DEN), N-nitrosopiperidine (NPd), N-nitrosopyrrolidine (NPy), and N,N'-dinitrosopiperazine (DNP); a substituted hydrazine: 1,2-dimethylhydrazine (1,2-(DMH); a mycotoxin: aflatoxin B_1 (AFB_1); and an aromatic amine, 2-aminoacetylfluorene] into metabolites that bind to cellular macromolecules including DNA.

The metabolism of BP has been extensively studied in explants of human bronchus cultured in a chemically defined medium. When compared to BP, binding values of its weakly carcinogenic analog, benzo[e]pyrene, were more than 100-fold less. Results from the trans addition of the 2-amino group of guanine to the 10 position of (+)7R,8S,9R,10R enantiomer of BP-7,8-dihydrodiol-9,10-oxide (BP diol epoxide I). Other minor adducts were also observed.

In a combined epidemiology-laboratory study we are comparing the metabolism of (BP) in cultured non-tumorous bronchial mucosa from cancer and non-cancer patients. Patient history and clinical findings are obtained from interviews and hospital records. Lung cancers are classified by cytochemistry and both light and electron microscopy. Macroscopically normal bronchial explants, collected at time of surgery or immediate autopsy, are maintained in chemically defined medium for 7 days. BP (1.5μ M) is then added for 24 hr. Binding levels of BP to mucosal DNA (pmoles per 10 mg DNA) are being measured as part of this study. In the initial 79 patients (21 without and 58 with lung cancer), the interindividual variation among samples from single cases is less than 2-fold. When compared to binding levels in non-cancer patients (18, mean; 2-82 range) higher levels are found in bronchi from patients with (a) epidermoid differentiated carcinomas including combined epidermoid-adenocarcinoma with a well-differentiated epidermoid component (34; 2-111; $p < 0.01$) or (b) mucous differentiated cancers with a non-glandular pattern (35; 11-96; $p < 0.025$), while no significant differences are found in bronchi from patients with glandular, mucous differentiated cancers (15; 1-45). Patient sex, race and history of tobacco smoking and consumption of alcoholic beverages are not confounding factors for the associations between binding levels and tumor types. Increased binding level was seen in patients with a family history of lung cancer. Trend analysis also indicates a decrease in level of binding with increasing age in the non-lung cancer group.

The extrapolation of carcinogenesis data among animal species depends in part on qualitative and quantitative differences between metabolic activation and deactivation of procarcinogens. Therefore, the metabolism of BP by cultured tracheobronchial tissues from different species -- human, bovine, hamster, rat and mouse -- has been investigated. The total metabolism, as measured by both organic solvent-extractable and water-soluble metabolites of BP, was substantial in the respiratory tract from humans and from animal species susceptible to the carcinogenic action of BP. The ratio of organic extractable metabolites to water-soluble metabolites was greater than one in hamster, human (patient with lung cancer), and C57Bl/6N mouse, but less than one in rat, bovine and DBA/2N mouse, suggesting that determination of both activation and deactivation pathways are important in assessing carcinogenic risk of a chemical. Tetrols and diols were the major organic solvent extractable metabolites. The level of *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene, the proximate carcinogenic form of BP, was three times higher in C57Bl/6N than in DBA/2N mouse trachea. *Trans*-9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene was the major metabolite formed by cultured hamster trachea. The binding levels of BP to cellular DNA were quite similar in all tissues, although slightly higher binding was observed in hamster trachea. Wide interindividual variation in the binding of BP to DNA was seen in tissues from outbred species. The major BP-DNA adducts in all animal species were formed by interaction of BP diol-epoxide with the 2-amino group of deoxyguanosine. Both stereoisomeric forms of (±)-(7β,8α)-dihydroxy-(9α,10α)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I) reacted with deoxyguanosine, the (7*R*)-form being the most reactive. No difference in the relative distribution of the various adducts was seen between the species except in the rat (CD, Wistar, and Buffalo) where BPDE-deoxyadenosine adducts accounted for 20% of the total modification. In cultured hamster trachea the persistence of the different adducts was similar. In conclusion, the metabolism of BP is qualitatively similar in tracheobronchial tissues from both humans and animal species in which BP has been experimentally shown to be carcinogenic.

A positive correlation in the binding level of BP between bronchus and colon ($r = 0.95$; $p < 0.001$) and duodenum ($r = 0.83$; $p < 0.001$) from the same individual (15 cases) was seen. No correlation in the binding level of BP and DMN to human bronchus was seen ($r = 0.38$; $p > 0.05$). The metabolic profile of BP in human bronchus differed quantitatively from that in colon and duodenum. A significantly greater amount of tetrols were formed by human bronchus, while less phenols were detected. Bronchus also more efficiently removed the primary metabolites to conjugated forms.

The major 7,12-DMBA-DNA adduct in cultured human bronchus was identified as formed between 3,4-dihydroxy-3,4-dihydro-1,2-epoxy-DMBA and the 2-amino group of guanine. Three other unidentified adducts were also detected.

The metabolism of BP was studied in both epithelial and fibroblast cells initiated from the same bronchus specimens. The total metabolism was 3-fold higher in the epithelial cells than in the fibroblast. No qualitative differences in the metabolic profile of BP between the explant culture and the epithelial cell cultures were observed. In the epithelial cells, the metabolic capacity remained high for at least 3 passages.

The 300-fold variation in the worldwide incidence of esophageal carcinoma suggests that environmental agents, including chemicals, cause this cancer. Since the interaction between chemical procarcinogens and human esophagus has not been previously studied, we examined the metabolic fate of BP, DMN and NPy in cultured non-tumorous esophagus from patients with and without esophageal carcinoma. Esophageal explants were cultured in a chemically defined medium for 7 days prior to adding [^{3}H]BP (1.5 M), [^{14}C]NPy (100 M) or [^{14}C]DMN (100 M) for 24 hrs. Radioactivity was found bound to protein (BP, DMN, NPy) and to DNA (BP, DMN). The major carcinogen-DNA adducts were 1) trans addition of (+) BP diol epoxide I at the 10 position to the 2-amino of guanine, and 2) with DMN, N-7 methylguanine and O-6 methylguanine (O-6 MeG/N-7 MeG= 0.3). The interindividual variations in binding levels to DNA were 99-fold for BP and 10-fold for DMN. The variation in binding levels among the 3 major anatomical segments (proximal, mid, distal) was less than 2-fold (2 cases). The metabolism of BP into water-soluble metabolites varied among the 8 patients from 1 to 68% of the total metabolism. The organic-extractable metabolites were similar in all patients. A model system for the study of carcinogenesis in the rat esophagus has been developed and the metabolism of a series of carcinogenic N-nitrosamines is being investigated in rat esophagus. After incubation of the explants with C-14 labeled nitrosamines [N-nitrosobenzylmethylamine (NBMA); DMN, NPy, N-nitrosoethylmethylamine (MEN)] at a concentration of 100 M for 24 hrs, the activation was determined by measuring ^{14}C-CO$_2$ formation and assaying the binding level to DNA. The highest binding levels to DNA were with NBMA, an organ-specific carcinogen for rat esophagus, and DMN. The oxidation of NBMA takes placesmainly in the benzyl-group as shown by the formation of benzaldehyde. The alkylation by the methyl group was about 10-fold higher than by the benzyl group. The predominant methylation products were O^{6}-MeGua and 7-MeGua (ratio 0.12). The ratio of O^{6}-MeGua/7-MeGua after incubation with DMN was 0.3. High levels of CO$_2$ were formed from DEN and 2-[^{14}C]MEN, indicating predominant oxidation of the α-position. Both α- and β-oxidation were observed in NPy, as identified by formation of 3-hydroxy nitroso pyrrolidine and 4-hydroxy butanol, respectively.

When the metabolism of BP in esophagus was compared to other organs from the same individual, no correlation in the level of binding to DNA was seen. The metabolic profile of the organo-soluble metabolite of BP was similar to that from human colon and duodenum, but the esophagus more efficiently conjugated the primary metabolites into water-soluble derivatives.

The metabolism of AFB, BP and DMN was studied in primary epithelial cell cultures from human esophagus. The level of binding of BP was several fold higher than that of AFB. The profile of BP metabolites was qualitatively similar to that of esophagus explants.

Significance to Biomedical Research and the Program of the Institute: Methodologies developed for and utilized in studies of carcinogenesis in experimental animals and cell cultures can be successfully extended to similar investigations in human cells and tissues. These investigations should eventually aid in identifying both chemical carcinogens and host factors determining susceptibility. Finally, these studies provide a much needed link between studies in experimental animals and man.

Proposed Course: Identify endogenous and exogenous factors that alter the metabolism of environmental procarcinogens. To continue combined laboratory-epidemiology study of carcinogen metabolism. To use ultramicroassays of carcinogen metabolism and carcinogen-DNA adducts so that metabolism and repair can be studied in biopsy specimens. To correlate metabolism of chemical carcinogens between different target tissues within a single individual. To compare the metabolism of chemical carcinogens in target tissues and possible "detector" cells, i.e., monocytes and macrophages. To continue comparative studies of carcinogen metabolism in cultured genomic distribution and modification of carcinogen-DNA adducts as well as their enzymatic repair.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04798-11 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Metabolism and Mode of Action of Vitamin A

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Luigi De Luca	Research Scientist	LEP, NCI
Other:	Tadashi Kurokawa	Expert	LEP, NCI
	Yoshihiro Shidoji	Visiting Fellow	LEP, NCI
	Carol Silverman-Jones	Microbiologist	LEP, NCI

COOPERATING UNITS (if any)
John R. Hassell, National Eye Institute
Kenneth Yamada, National Cancer Institute

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Differentiation Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.5 .	PROFESSIONAL: 2.5	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A mannosyl transferase system specific for retinylphosphate as a glycosyl carrier in rat liver microsomal membranes was characterized. The system was active and specific in the absence of detergent, under conditions which preserved the intactness of the membrane. Arrhenius plots of mannosyl-retinylphosphate (MRP) synthesis revealed two slopes with a transition temperature at 12°C. The biologically inactive analog perhydroretinylphosphate (pRP) formed MpRP but this reaction had a single slope. RP, but not pRP, was active in protein mannosylation. SDS-PAGE revealed major bands at 15, 30, 45, and 60 thousand daltons. The inactive analog pRP formed MpRP: this mannolipid was still available to the enzyme for reversal by excess GDP. On the contrary, the active compound MRP, once synthesized, was no longer available for reversal unless detergent was added along with GDP. It is concluded that RP acts as a direct carrier of mannose in the biosynthesis of specific glycoproteins and that saturation of the double bonds in the polyenic chain of vitamin A renders the molecule inactive in this process.

Objectives: To find and characterize the specific mannosyl transfer system of enzymes that utilize retinylphosphate as a glycosyl carrier in mammalian membranes.

Methods Employed: In addition to procedures which have been described in the literature, the following methods have been developed.

Filter Assay for Mannosyl Transfer Reaction: The standard incubation was conducted as follows: 0.4 μCi of GDP-[^{3}H]mannose (or GDP-[^{14}C]mannose) and 10 μg of retinylphosphate in 99% methanol were transferred to test tubes. After removing the solvent under a nitrogen stream, 4 mg/ml bovine serum albumin (BSA), GDP-D-mannose (24 μM final), 30 mM Tris-HCl buffer (pH 8), 2.5 mM $MnCl_2$, 8 mM NaF, 2 mM ATP, 5 mM AMP and 0.9 mg of the microsomal protein were added in a final volume of 200 μl. The mixture was incubated at 37°C for 2 min. The reaction was stopped by addition of 1 ml ice-cold medium A and immediately poured on the MF-millipore (HA 0.45 μm) on the filter manifold (Millipore), allowing processing of several samples. The filter was washed with an additional 1 ml of medium A to remove unbound radioactive materials. The radioactivity retained on the filter was measured in 10 ml of Aquafluor (New England Nuclear); counting efficiency for ^{3}H was 30% and ^{14}C, 70%.

Protein A-Sepharose Column Chromatography. After 2 minutes incubation of GDP-[^{3}H]mannose (0.1 Ci) with 0.15 mM RP in the BSA-rat liver microsomal system, the radioactive compounds on the Millipore filter were extracted with 5 ml of chloroform:methanol (2:1) twice. The resultant extract was applied to DEAE-Sephacel, equilibrated with 99% methanol, to remove the excess unreacted RP (Sasak et al., 1979). After removing the solvent under N_2-stream, the 10 mM ammonium acetate eluate was incubated with anti-vitamin A rabbit antiserum (0.5 ml) in the ice-box for 20 min. A portion of the mixture was applied to a column (0.8 x 1.5 cm) of Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden), equilibrated with 30 mM Tris-HCl buffer, pH 8.0 containing 0.1% Triton X-100. After washing the gel with 5 volumes of the starting buffer, the radioactivity retained on the column was eluted with 10 M retinoic acid in the buffer. As a control experiment, non-immune rabbit serum (GIBCO,NY) was used instead of anti-vitamin A serum. Fractions of 10 drops were collected and radioactivity in each fraction was measured in 10 ml of Aquafluor.

Major Findings:

Mannosylretinylphosphate (MRP) Synthesis in the Absence of Detergent. These studies have shown that the transfer of mannose from GDP-mannose to retinylphosphate occurs with a very high efficiency in incubations of rat liver microsomal vesicles in the presence of BSA and without detergent. The sugar transfer to microsomes (mostly accounted for by MRP synthesis at two minutes of incubation) was highly specific for retinylphosphate and for GDP-mannose as a sugar donor in this assay system. Neither non-phosphorylated retinoids nor phospholipids were able to stimulate the mannosyl transfer process, suggesting that the reaction requires both the retinol and the phosphate moiety in the RP molecule. RP does not function as a glycosyl acceptor from UDP-[^{14}C]-glucose, UDP-[^{3}H]galactose, GDP-[^{14}C]glucose, UDP-N-acetyl[^{14}C]glucosamine and UDP-N-acetyl-[^{14}C]galactosamine, consistent with previous results.

Moreover, the detergent Triton X-100 stimulated the formation of dolichylmannosylphosphate, but markedly lowered MRP synthesis in the BSA-RP incubations. Therefore, we conclude that RP-mannosyl transferase is highly sensitive to detergents, whereas Dol-P mannosyltransferase appears to be activated by Triton X-100, which may also facilitate solubilization of Dol-P. We have not excluded the possibility that the detergent might also inhibit the formation of a putative BSA-RP complex which may be necessary for the reaction.

The amount of MRP formed increased from 5 picomoles/min/mg protein in the presence of the detergent to 520 picomoles/min/mg protein in the absence of the detergent. However, the Km for GDP-mannose was found to be similar (18 μM) to that (13 μM) found in the presence of detergent. These data would therefore suggest that the detergent effect on RP-mannosyltransferase is due to a decrease in the number of active enzyme molecules. Specific antibodies to vitamin A were able to recognize the -ionone ring in the molecule of MRP, thus confirming that structure.

Arrhenius Plots and Irreversibility of Mannosyl Retinylphosphate Synthesis. The Arrhenius plot of the reaction RP + GDP-mannose → MRP + GDP (Reaction 1) was obtained utilizing radioactive GDP[^{14}C] mannose and the filter assay for MRP detection. A transition temperature for this mannosyl transfer process was evidenced at 12°C by this graphic method, indicating that the process is physically complex. The inactive analog perhydroretinylphosphate (pRP), though active as an acceptor of mannose, displayed a monophasic mode without transition: its lack of activity in catalyzing mannosyl transfer to protein may well be related to the lack of a transition temperature. RP is instead active in mannosyl transfer to protein. Further, reversibility of reaction 1 by excess GDP did not occur with RP, while with pRP it was easily observed. Reversal of the RP-mediated reaction 1 was seen only when detergent was included in the incubation.

These results suggest that, once synthesized, mannosylretinylphosphate is no longer available to the mannosyl transferase unless detergent is added.

Transfer to Protein. Protein mannosylation was observed in incubations containing RP. Without RP or in the presence of pRP, protein mannosylation was not evident. SDS-PAGE revealed acceptors for mannose in the RP incubation at 15, 30, 45 and 60 thousand MW.

Significance to Biomedical Research and Program of the Institute. It is the aim of this project to investigate the mechanism(s) by which vitamin A functions in the body. Inasmuch as vitamin A and its derivatives, the retinoids, are active as preventive agents of epithelial cancer, such investigation may yield useful information on mechanisms whereby normal tissue-specific phenotypic expression is maintained by retinoids.

A substantial body of work has shown a biochemical involvement of vitamin A at the level of the biosynthesis of glycoproteins. The phosphorylated vitamin appears to function in mammalian membranes as a carrier of mannosyl residues destined to specific glycoproteins. Some glycoproteins mediate cell to cell recognition and adhesion (e.g. fibronectins); others have hormonal functions in tissue growth and development (e.g., the gonadotropins); others display epithelioprotective functions (e.g., secretory mucins); thus, it is reasonable to propose that the newly found involvement of the phosphorylated vitamin in glycoprotein biosynthesis may explain its effect on mucus secretion, adhesion and the maintenance of normal phenotypic expression.

Proposed Course: 1) To investigate whether the transition temperature in the mannosyl carrier function of retinylphosphate is also observed when Arrhenius plots are made with membranes from neoplastically transformed cells. 2) To further characterize the membrane glycoprotein acceptors and to study their location in the bilayer by utilizing a variety of glycosidase enzymes. 3) To work out an in vitro assay for retinol kinase. 4) To investigate the possibility that double bond isomerization is involved in the carrier function of retinylphosphate.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05092-03 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathology of Tumors and Other Lesions Induced in Experimental Animals by Prenatal or Postnatal Exposure to Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.M. Rice	Section Chief	LEP NCI
Other:	P. Roller	Research Chemist	LCM NCI
	W.T. London	Section Chief	NINCDS NIH
	A.E. Palmer	Veterinary Director	LEP NCI

COOPERATING UNITS (if any)

NINCDS
LCM, NCI

LAB/BRANCH Laboratory of Experimental Pathology

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Selected direct-acting and metabolism-dependent compounds are administered by various routes to non-pregnant and to precisely timed-pregnant rodents and non-human primates, and the treated animals and their offspring are followed for subsequent tumor development. Neoplasms which develop are classified by light and electron microscopy, histochemistry, transplantation, and cultivation in vitro. Dose/response relationships are precisely quantitated to establish age-dependent qualitative and quantitative differences in susceptibility to different types of carcinogens in different organ systems.

Objectives: To explore and define the varying susceptibilities of different organ systems in rodents and primates to direct-acting and metabolism dependent carcinogens during prenatal and postnatal development; and precisely to characterize neoplastic and selected non-neoplastic conditions by light and electron microscopy, histochemistry, transplantation, explantation to cell and organ culture, and by other procedures as required.

Methods Employed: Carcinogenic chemicals specifically selected or designed for a given study are purified after purchase, or synthesized de novo and characterized thoroughly by chromatographic and spectroscopic procedures. Radiolabeled compounds are similarly prepared as required. Carcinogens in precise dosages are administered to non-pregnant or exactly timed-pregnant rodent (rat, mouse) or non-human primate (patas or rhesus monkey) subjects, and the treated animals and their offspring are followed carefully for tumor development. Surgical procedures are devised as necessary to modify the processing of carcinogens (e.g., bile duct cannulation) in studies on organotropism of carcinogens or to remove single operable growths from primates in order to prolong life and allow further observation for additional tumor development. Selected neoplasms and fetal tissues are explanted to cell culture and to athymic (Nu/Nu) mice for further study of their properties. Tumors are carefully classified histogenetically by light and electron microscopy and by histochemistry.

Major Findings: Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in the Old World monkey, *Erythrocebus patas*, have been continued and expanded. Additional cases of both mesenchymal and epithelial tumors were observed in the offspring of monkeys given ENU intravenously during pregnancy, especially when treatment was begun during the first month of gestation. These additional findings confirm the tentative conclusions previously drawn that, like rodents, this representative primate species is quantitatively more susceptible to the direct-acting alkylating agent ENU during prenatal life, with animals exposed *in utero* exhibiting a higher incidence of tumors after a shorter latency than juvenile or adult animals given the same dose directly.

Careful morphologic evaluation of tissues from female patas monkeys that died of a widely disseminated, hemorrhagic malignancy within several months of the beginning of exposure to ENU during pregnancy has confirmed the provisional diagnosis of gestational choriocarcinoma, marked by replacement of endometrial stroma by tumor cells, the occlusion of numerous blood vessels in the uterine myometrium by masses of tumor cells morphologically indistinguishable from intravascular trophoblast, and numerous distant metastases, principally to the lungs. The presence of tumor tissue within the placenta in one case constitutes additional evidence for the trophoblastic origin of the neoplastic cells. This observation provides the only animal model of systemically inducible trophoblastic malignancy.

Histologic evaluation of tumor tissues from the offspring of rhesus monkeys (*Macaca mulatta*) given ENU during pregnancy has shown that the most common inducible tumor, a massive, lethal primary pulmonary mass that grossly and

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biologically resembles the pulmonary sarcomas previously recorded in transplacentally exposed patas monkeys, is a unique embryonal tumor of mixed epithelial (bronchial) and mesenchymal cell types. Sections of this tumor strongly resemble the histology of the lung during the early stages of its morphogenesis. The rhesus tumor is unlike any neoplasm of man or of common small experimental species. In addition to extending to a second primate species the generalization of enhanced susceptibility to carcinogenesis during prenatal development, this finding emphasizes the possibility of unique, species-specific responses even to metabolism-independent carcinogens. It also emphasizes the impossibility of a priori extrapolation between species with respect to the nature and anatomic localization of neoplasms that result from exposure to a given carcinogenic substance.

A study on the pathogenesis of renal lesions that develop in rats chronically dosed by gavage with the flame retardant tris (2,3-dibromopropyl) phosphate also demonstrated the induction of colonic mucosal tumors by this agent for the first time.

Studies have continued on the biological effects of the α-acetoxy derivatives of dimethylnitrosamine (DMN-OAc). In the course of an experiment originally designed to define optimal conditions for investigation of cell cycle dependence of hepatocellular carcinogenesis in rats, DMN-OAc or methylnitrosourea (MNU) were given to partially hepatectomized rats as a single injection into the portal vein. An unexpectedly large fraction of the MNU passed through the liver remnants, inducing tumors in many distant organ systems in high incidence, including the kidneys, intestinal tract, testicular mesothelium, nervous system, and other sites. A completely unexpected finding in the MNU study was 29 odontogenic tumors in 134 treated F344 rats. These tumors appear to arise near the roots of the continuously erupting incisor teeth. Ninety percent were complex odontomas, consisting of well-differentiated but haphazardly arranged cementum, dentin, enamel and pulp. Dentin comprised the bulk of the tumor masses. In contrast, the remaining tumors (3 cases) were largely ameloblastic. This chance finding contributes a useful new model for the induction of such tumors in high yield, which are of potential use in the developmental biology of neoplasia; complex odontomas are composed of tissues derived from two germ layers, dental ectoderm and mesoderm, which must cooperatively exert inductive influences on each other in order to differentiate sequentially to form the extracellular products, dentin and enamel. In contrast, DMN-OAc, although dependent on intracellular esterase for its activation to a reactive ultimate carcinogen, gave rise to a significant incidence of primary liver cell tumors and to few tumors in other organ systems, suggesting that it might be a useful agent for the study of cell cycle dependence of susceptibility to carcinogenesis in partially hepatectomized animals.

Significance to Biomedical Research and the Program of the Institute: Research on animal models of human childhood neoplasms should provide an insight into the types of causative agents and modes of exposures responsible for childhood cancer. It is to be expected that natural selection would tend to eliminate genotypes in the human population which predispose individuals to the development of fetal neoplasms before attaining reproductive age, yet the incidence

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of tumors in childhood is relatively constant. Epidemiological studies have pointed to the occurrence of childhood neoplasms in association with certain types of congenital malformation which are non-inherited and suggest that environmental agents, alone or in combination, may play a role in the induction of such neoplasms. The inducibility of tumors very similar to the pediatric tumors of man by chemical carcinogens in laboratory rodents and primates further supports this view.

Most tumors induced transplacentally in rodents are of adult types and appear during adult life in individuals exposed in utero, resembling human experience with diethylstilbestrol. The ENU studies in monkeys have provided experimental data indicating that both adult and pediatric tumor types develop in at least one species of primate in response to carcinogenic exposure in utero and suggest that chemical carcinogens may be involved in the prenatal genesis of pediatric and possibly certain adult types of tumors in man. The demonstration of the inducibility of uterine choriocarcinoma by chemical carcinogens, at low exposure levels, further illustrates the importance of preventing human exposure to carcinogenic chemicals during pregnancy in either the workplace or environment.

Carcinomas of the gastrointestinal tract and pancreas comprise the largest source of cancer morbidity in the adult population of the United States. The discovery, both in a particular strain of mice and in a representative non-human primate, that intestinal tumors are among the results of prenatal exposure to direct acting chemical carcinogens raises the possibility that tumors of this sort may result not only from postnatal exposure to low level carcinogens over a long period of time, but may develop from a transient and even a single exposure to a potent carcinogen during a particularly susceptible period of development. Factors modifying the biology and evolution of these tumors of lining epithelia should be studied in this context.

Proposed Course: Monkeys exposed to ENU or DEN either transplacentally or directly (after weaning) should continue to be observed for the development of tumors. In vivo studies should emphasize further refinement of definition of periods of maximal prenatal susceptibility to direct-acting vs. enzyme-activated transplacental carcinogens; and the extent to which the enzymes, which activate different classes of metabolism-dependent carcinogen, can be induced by either the carcinogens or other agents in fetal, maternal and placental tissues at different stages of prenatal development. The possibility that the phenomenon of tumor promotion can be demonstrated in primates prenatally exposed to ENU, either by increasing the incidence of hepatocellular tumors by postnatal exposure to phenobarbital, DDT, etc., or by increasing the incidence of skin tumors by topical application of phorbol esters, will be explored. Pharmacodynamic studies will be continued to further study maternal-fetal distribution and tissue/organ localization of ENU-^{14}C in this species in comparison with rats and mice.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05093-03 LEP

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Differentiation of Fetal Cells and Tissues: Modulating Factors in Organ-specific Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Rice	Section Chief	LEP	NCI
Other:	W. Edwards	Visiting Scientist	LEP	NCI
	P. Donovan	Chemist	LEP	NCI
	A. Perantoni	Microbiologist	LEP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Experimental Pathology

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.5	PROFESSIONAL: 0.5	OTHER: 1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The roles of morphogenetic differentiation in controlling the phenotypic expression of neoplastic transformation, the degree of malignancy of tumors, and the susceptibility of developing organs to carcinogenesis are studied using organ culture and tissue transplantation techniques, with current emphasis on the kidney.

Objectives: To identify and characterize those aspects of morphogenetic differentiation which modify the consequences of prenatal exposure to chemical carcinogens, especially in the nervous and genitourinary systems. To modify the biological behavior of chemically induced tumors, including their rate of growth, capacity to invade, and ability to metastasize. The ultimate objective is to elucidate the control of expression of the neoplastic phenotype in transformed cells.

Methods employed: Short- and long-term cell and organ culture techniques are developed, and the features of tissue rudiments maintained therein characterized by histochemical, light microscopic, and ultrastructural techniques. Cultures of both normal fetal organ rudiments and selected tumors are utilized to explore the effects of morphogenetic differentiation and its induction on the behavior of tumors of undifferentiated cell type, including nephroblastic tumors of the kidney. Tumors are induced transplacentally or by direct treatment in experimental animals to provide suitable material for study and are transplanted serially in appropriate recipients to develop standard, manipulatable models for studies *in vitro*. Surgical procedures relating to tissue transplantation are adapted as necessary to study the capacity of various recipient sites to modify differentiation of selected transplantable tumors. Substances and tissues known to influence differentiation are combined with tumors and normal undifferentiated organ rudiments in organ culture and their effects on differentiation of normal and neoplastic tissues characterized. All studies are performed in more than one species, selecting species (such as the rat and mouse) which can be readily manipulated in the laboratory and in which responses of selected organ systems, such as the kidney and brain, to chemical carcinogens during fetal life vary extremely both in quantitative responses to chemical carcinogens and in the nature of tumors induced.

Major Findings: Previous reports have described our development of procedures for culturing fetal organ rudiments and for producing primary and established monolayer cultures of fetal and adult epithelial organs.

A continuing effort to manipulate morphology and differentiation of normal and neoplastic renal tissue through media additives is under way. Mouse kidneys develop purely epithelial, well-differentiated tumors of adult type in response to transplacental carcinogens, while rat kidneys generate adult epithelial, nephroblastic, and undifferentiated (blastemal cell) tumors; the ability of rat and mouse renal tumor cells to respond to differentiation signals and the influence of the latter on their morphology, growth, and biologic behavior are being investigated. Work has continued on maintaining kidney rudiments in organ culture. Metanephric rudiments for organ culture were obtained from 12-13 day C57BL/6N mouse fetuses and from 14-15 day Fischer, F344, rat fetuses and cultured to study the effects of hormones and culture conditions on survival and differentiation *in vitro* in order to optimize conditions for manipulation of renal differentiation. Each kidney rudiment was placed on a gelfoam sponge in Williams' Medium E supplemented with hydrocortisone (HC, 5 μg/ml), insulin (I, 5 μg/ml), and fetal bovine serum (10%). By light and electron microscopy, tubulogenesis was most pronounced 2-5 days (mouse) or 7-9 days (rat) after explantation. Mitotic activity occurred in undifferentiated cells and in nephrogenic vesicles but was most frequent in differentiating tubular epithelium. Occasionally, well-differentiated but avascular glomeruli were seen. Explants survived for 15 days with HC plus I, but only 10 days in control media. Both rat and mouse explants cultured with HC plus I formed increased collagen and glycogen compared with explants cultured in unsupplemented media or media containing only one hormone. γ-Glutamyl transpeptidase, a marker enzyme of kidney proximal tubular epithelium, was localized in cells involved in tubule formation in organ culture and *in vivo*. Under these conditions, HC plus I significantly enhance survival, growth, and differentiation of explanted rat and mouse kidney rudiments.

Experiments are also in progress in which natural (ureteric bud) and heterotopic (fetal spinal cord) inductive tissues will be used as potential inducers of morphogenetic differentiation of "blastemal-cell" (undifferentiated) renal tumor tissues.

Renal tumors can be induced in mice by exposure to a variety of chemical carcinogens. The induced tumors are invariably well differentiated and of adult epithelial morphology (Lombard et al., *J. Natl Cancer Inst.* 53:1677-1685, 1974). By transplanting the renal blastemas of 12-15 day 129/SV mouse fetuses to the testes of adult mice of the same strain, however, Javadpour et al. (*J. Urology* 107:931-937, 1972) claimed to have produced tumors developmentally and histologically similar to human Wilms tumor. Histologically, the lesions produced were asserted to have consisted of sarcomatous components with embryonal elements, malignant stromal tissue and epithelial components.

In an effort to confirm and extend the results of Javadpour et al., the renal blastemas of 12 day 129 S/V mouse fetuses were transplanted by their procedure to the testes of adult 129 S/V mice. Further, in order to evaluate the influence of host and donor strains in this model system, the renal blastemas of 12 day C57BL/6N or (C57BL/6N male x 129 S/V female)F_1(B6129F_1) mouse fetuses were transplanted to the testes of the respective adult syngeneic host. Additionally, B6129F_1 mice served as transplant recipients of 12 day 129 S/V or C57BL/6N mouse fetal kidney.

The transplants are currently being examined histologically and histochemically; preliminary results indicate that this transplant system is not a potential model for Wilms tumor (nephroblastoma) induction in mice. Metanephric rudiments that survived in the ectopic graft site matured into functioning ectopic kidneys; no tumors were produced.

Undifferentiated (blastemal cell) tumors induced by the carcinogen methyl-(methoxymethyl) nitrosamine in newborn F344 male and female rats have been successfully transplanted to syngeneic recipients; and of the 27 primary tumors initially transplanted, 3 of the 16 tumors that grew intradermally evolved into noninvasive, rapidly growing and initially completely undifferentiated tumors which strongly resemble undifferentiated renal blastema in their morphologic features. Previous reports have described the maintenance of fragments from these tumors in organ culture. Fragments from transplant tumors, including the three permanent lines, were studied in organ culture, generally with maintenance of their highly cellular, undifferentiated pattern of growth. Under certain circumstances, these tumors have occasionally, and without additional manipulation differentiated to form structures in organ culture indistinguishable from those seen during early tubulogenesis in the permanent kidney of the fetal rat. This finding suggests that the experimental materials are now at hand for systematic study of factors influencing differentiation of normal and neoplastic renal blastema originating from the rat and for comparing it to comparable tissues in the mouse in which differentiation, as judged from the types of tumors seen, is much more efficient. These tumors were devoid of any evidence of epithelial differentiation by histochemistry and by light and electron microscopy through the first five transplant generations.

One of the three transplant tumors began to form papillary (epithelial-like) structures in vivo after the 6th transplant generation (intradermal transplants). The number of "differentiating areas" (papillary-like structures) increased with each transplant generation from the 7th to the 19th generation. Studies on the 20th and subsequent transplant generations are in progress. Generally both the undifferentiated and the "differentiating areas" (papillary structures) in this tumor were well maintained in organ culture. Histochemical and electron microscopic characterization of these tumors are in progress. The epithelium lacks morphologic and biochemical features of renal tubular epithelium, however.

Significance to Biomedical Research and the Program of the Institute: Studies in rodents have shown that a fetus may be as much as two orders of magnitude more susceptible to carcinogens than an adult of the same species, strain, and sex. The precise reasons for this enhanced vulnerability are not clearly understood, and the fact remains unexplained that, in rodents, carcinogens acting on differentiating fetal tissues induce principally tumors of adult epithelial morphology. Many tumors that develop as a consequence of transplacental exposure to carcinogens are morphologically identical to those inducible in adults. In the mouse kidney, for example, only adenomas and a few carcinomas originating from proximal convoluted tubules develop after prenatal exposure to carcinogens when the kidney is mostly undifferentiated. This suggests that the fundamental genetic damage inflicted on undifferentiated fetal cells does not preclude subsequent programmed differentiation. The fact that differentiation overrides expression of neoplastic transformation in a given organ system (e.g., the kidney) of certain species such as the mouse, but does not do so in others, such as the rat, provides a route to exploration of the basic nature of cellular differentiation to the control of neoplastic growth, in the context of prenatal susceptibility to carcinogens.

Proposed Course: Rat renal "blastemal-cell" tumors will continue to be studied in transplantation and in cell and organ culture to determine whether the morphologically undifferentiated tumors can be induced to form characteristic epithelial structures resembling renal tubules, and whether the enzymes characteristic of renal epithelium will develop as markers of morphologically demonstrable differentiation. In organ culture, both natural (ureteric bud) and heterotopic (fetal spinal cord) inductive tissues will be used as potential inducers as well as chemical agents which are known to affect other in vitro systems in which morphogenetic differentiation occurs (cAMP, IUDR, DMSO). The goal of this program is initially to determine whether the lesser tendency of fetal rat kidney (in comparison with that of the mouse) to form differentiated epithelial tumors after exposure to transplacental carcinogens is due to interspecies differences in cellular responsiveness to mediators of morphogenetic differentiation.

A major characteristic of the response of rodents to chemical carcinogens during intrauterine development is that susceptibility to neoplastic transformation generally is not demonstrable prior to completion of definitive organogenesis, which marks the beginning of the fetal period of development. True embryos, in which undifferentiated tissues are only beginning to form identifiable organ rudiments, are subject to teratogenic damage but are not, in general, at risk for subsequent tumor development as a consequence of exposure to carcinogens during this stage of development. The question arises whether this indicates that potential neoplastic transformants are generated, but are prevented from expressing their neoplastic genotype phenotypically by proliferation to generate a tumor. The latter might be accomplished through cell-cell interactions or other mediators of programmed normal differentiation to which cells altered by carcinogens are still responsive. A future project is planned using the fetal hamster in cell cultures from which morphologic transformation of mesenchymal cells is demonstrable. Prenatal hamsters will be subjected transplacentally to a carcinogenic dose of metabolism-independent carcinogen at different stages of development, from implantation of the blastocyst through late fetal life. Cultures prepared from the conceptuses thus exposed will be studied for the presence of transformed cells in an effort to demonstrate the presence of latent transformed cells in fetal tissues that appear refractory to carcinogenesis during early development. The fact that transformation is readily demonstrated in fetal hamster fibroblasts that originate from the soft connective tissues in which tumors are not seen following transplacental exposure to carcinogens strongly suggests that such an approach will be fruitful.

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PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Carcinogenesis Studies Using Cultures of Human Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John Lechner	Expert	LEP	NCI
	Aage Haugen	Guest Worker	LEP	NCI
	Curtis C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP	NCI
Other:	Herman Autrup	Senior Staff Fellow	LEP	NCI
	Susan Schlegel	Expert	LEP	NCI

COOPERATING UNITS (if any)

University of Maryland, School of Medicine, Baltimore, MD; Litton Bionetics, Inc., Kensington, MD; Georgetown University, School of Medicine, Washington, D.C.

LAB/BRANCH

Laboratory of Experimental Pathology (LEP)

SECTION

Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.5	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Developed defined methods to grow replicative cultures of normal human bronchial epithelial cells either without serum or Swiss mouse 3T3 feeder-cells. These cells can be subcultured several times, will undergo 35 population doublings and have the expected epithelial cell characteristics of keratin, desmosomes and blood group antigens. Further, mitotically quiescent cells will differentiate into cells with beating cilia, a characteristic of normal bronchial epithelium. Dissociated single cells form colonies when plated at low density. In vitro carcinogenesis experiments with normal bronchial epithelial tissue and cell cultures have yielded populations of cells which have abnormal characteristics. These phenotypically altered cells (PACS), which have the keratin epithelial cell markers, have extended population doubling potentials, abnormal human karyologies and abnormal serum and growth factor requirements. The tumorigenic potential of these isolates are being assessed in athymic nude mice.

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(Rev. 10-76)

Objectives: To develop systems to study malignant transformation of human epithelial cells. These studies include the following: (1) developing replicative epithelial cell cultures from bronchial tissue; using defined media; (2) evaluating both long-term explant and rapidly dividing cell cultures as model systems to study *in vitro* malignant transformation; (3) studying the effects of chemical and physical co-carcinogens and promoters on the progression of phenotypically altered cells to malignancy; and (4) evaluating carcinogenic exposure of xenotransplanted subsegmental bronchi as a model system to study human carcinogenesis.

Methods Employed: Human bronchial tissues are obtained from surgery, medical examiner, and "immediate" autopsy donors. Bronchial tissues are dissected from surrounding stroma, are cut into 0.5 cm square pieces and then used to establish explant cultures.

Two types of long-term explant cultures are being used to scrutinize cellular changes induced by exposure to chemical carcinogens. Quasiquiescent long-term explant cultures maintained for more than 1 year and exposed to 4-nitroquinoline-1-oxide (4NQO), both with and without asbestos, are examined periodically for foci of mitotic epithelial cells. Further, to elucidate the interaction between epithelial cells and asbestos, cells have been grown onto grids and then exposed to asbestos for 3 weeks. These cells are being studied using high-voltage and scanning electron microscopy techniques. Explant cultures are also used to study carcinogenesis in a continually growing epithelium. After epithelial cell migration, the explant is transferred to a new dish. Both the outgrowth and transplanted explant dishes are maintained. After a new outgrowth, the explant is again transferred. This protocol is repeated for a period of 1 year. The explants are repeatedly exposed to either 7,12-dimethylbenz[a]anthracene, DMBA (weekly) or 4NQO (biweekly).

Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. As many as 20 successive outgrowth cultures can be obtained from a single tissue by repeated transferring of the explant. Upon transfer of the explants to new dishes, the outgrowth cultures are incubated in defined, serum-free medium to expand the population, then subcultured. These cells are then used in *in vitro* carcinogenesis, cell nutrition, carcinogen metabolism, and DNA repair experiments or cryopreserved for future use.

Several criteria are being used to establish the normal and epithelial phenotype of the cells grown in culture. Establishing markers to identify normal epithelial cells *in vitro* has been an important objective for this laboratory. These efforts have identified several criteria, including karyology, polygonal morphology, ciliary activity, scanning electron microscopic morphology, ultrastructural identification of tight junctions, desmosomes and tonofilaments, production of acidic and neutral mucopolysaccharides, and immuno-staining of keratin, blood group antigens and type IV collagen, population doubling potential, clonal growth rate and mitogenic responsiveness to peptide growth factors and hormones.

Changes in cellular properties, as well as abnormal exfoliative cytology and anchorage independence, are being sought as a result of exposure to chemical carcinogens. Growth in athymic nude mice is being tested using several procedures. Cells are being injected both subcutaneously and intracranially into untreated or either estradiol or antilymphocytic serum-treated nude mice. Further, freeze/thawed rat trachea are being used as containers. Cells are injected into the trachea lumen, the ends are tied-off, and the trachea are implanted subcutaneously. The lumen provides a pseudo-basement membrane for cell attachment.

As a model system for in vivo carcinogenesis, small sub-segmental bronchi are implanted subcutaneously into nude mice. After vascularization, carcinogens incorporated into beeswax pellets, absorbed on lycra fibers or in gelatin pellets, are inserted into the lumen. Periodically, these tissues are recovered and assessed by pathological examination.

Major Findings: Human bronchial epithelial cell culture experiments have yielded the following results: (1) a method for routinely initiating replicative epithelial cell cultures of human bronchus has been developed. Large pieces of bronchus tissue are initially set-up as explant cultures and incubated in a rocking chamber for two to three days to facilitate removal of mucus. These explants are then cut into smaller pieces and explanted into Ca^{2+} reduced, M199 medium containing 1.25% fetal bovine serum. This medium, with a low serum concentration, permits rapid outgrowth of epithelium but retards growth of the fibroblastic cells. Thus, after 5-10 days incubation, very few fibroblastic cells are present among the epithelial outgrowth. At this time, the tissue explant is transferred to a new dish for reseeding a second wave of epithelial cell outgrowth. Sequential tissue transfer has been repeated up to 20 times, over a period of one year, and the epithelial cells were not discernably different from the first outgrowth culture; (2) a defined (serum-free) medium was developed. Clonal growth doseresponse experimentation was used to tailor the concentrations of nutrients and growth factors in MCDB 151 medium to meet the requirements of the normal human bronchial epithelial cells. Modified MCDB 151 medium (151) contains transferin, insulin, epidermal growth factor, cholesterol, hydrocortisone, and ethanolamine. In addition, the Ca^{2+} and SO_4 levels were adjusted. Supplementation with as little as 0.25% serum results in a decrease in clonal growth rate; 8% supplementation completely inhibits growth; (3) further improvements in in vitro conditions and techniques were obtained by modifying the surface of the petri dish and incorporating urea into the subculturing methodology. Clonal growth-dose response experiments showed that precoating the surface of the dish with a mixture of collagen and fibronectin improved the rate of cell attachment, plating efficiency and clonal growth rate. In addition, plating efficiency was improved by prewashing explant outgrowth cultures with 0.25 M urea prior to trypsin and EGTA dissociation; (4) incorporation of 1.25% serum into M151 altered the Ca^{2+} requirement. Whereas no significant change in the Ca^{2+} requirement was noted between 60 and 1000 M in serum-free medium, 50% inhibition of growth occurred above 700 M Ca^{2+} with serum supplementation. The calcium concentration of the medium influences colony morphology and cell shape. The cells are small and tightly polygonal when growing in normal (1 mM) levels of calcium. Progressive reduction of the calcium concentration to 0.06 mM causes the cells to become flatter and less closely associated; (5) EGF decreases the number of desmosome junctions between cells. Examination of in situ fixed epithelial colonies revealed markedly less desmosomes, compared with colonies grown without EGF; (6) serum influenced growth factor requirements. Cholera toxin and transferin were growth stimulatory in media supplemented with 1.25% serum. The absence of serum obliterated cholera toxin and transferin stimulation; (7) a less-defined method to grow human bronchial epithelial cells was developed prior to elucidating serum-free conditions. In this system, Swiss mouse 3T3 feeder-cells are added to the cultures and the cells are grown in Ca^{2+} reduced M199 medium supplemented with epidermal growth factor (EGF), cholera toxin and 1.25% serum. Coating the dish surface with fibronectin improved clonal plating efficiency (from 2 to 15%) and increased the clonal growth rate (2.5-fold). The addition of other factors, e.g., MSA, FGF and EGGS were less mitogenic; (8) cultures of human bronchial epithelium have the normal human karyotype and average 35 population doublings in vitro; (9) epithelial cell markers have been demonstrated. Keratin was shown by both immunospecific, Kreyberg and rhodamine B staining. Blood group antigens were also detected by immunospecific staining. However, mucopolysaccharide staining was negative. Quiescent cultures differentiated into cells have beating cilia. Ultrastructurally, these cells have numerous tonofilaments and desmosomes; (10) normal epithelial explant and cell culture systems for adult bronchial epithelium have now been extensively developed. Thus, _in vitro_ chemical carcinogenesis experiments have become potentially feasible. Large quantities of epithelial cells can be readily obtained permitting experiments using large numbers of cells at risk to be repeated several times.

Carcinogenesis experiments have yielded the following: (1) explant cultures were maintained for more than 1.5 years. A steady state condition was established whereby dying cells were replaced by cells migrating from the tissue. As time progressed, the outgrowth cultures become predominantly fibroblastic in appearance. After 1.5 years, these explant tissues were transferred and a new wave of mitotic epithelial cells migrated from the tissue, attesting to long-term viability of tissue. However, the population of epithelial cells was judged not to be phenotypically altered since normal sinescence ensued after few successive subculturings; (2) phenotypically altered cells (PAC) were isolated after repeated treatments of bronchial epithelial cells with Ni^{2+} or DMBA. PACs seemingly arose via two pathways. Ni^{2+}-induced PACs arose as individual clones. These clones appeared after the exposed semi-confluent cultures had ceased division. Three to seven weeks after the cultures had become mitotically quiescent, an occasional cell began to divide and grew into a colony. Twelve of the PAC colonies have been isolated from three experiments and expanded. The PAC culture which arose after 6 months of repeated exposure of explants to DMBA apparently developed via a different mechanism. Clonal origin cannot be suggested; escape from senescence developed throughout the outgrowth cell population and a period of mitotic quiescence did not precede their appearance; (3) five PAC cultures, which arose in different experiments and are autopsy donor unrelated, have been partially characterized. All express the keratin epithelial cell markers and exhibited desmosomes in early passage. Chromosomal studies revealed both normal and anaploid karyologies. The clonal growth response to ethanolamine and serum and Ca^{2+} differ both among themselves and as compared with the normal cells; (4) the tumorigenicity of these isolates has been tested in nude, athymic mice. Rat tracheal container experiments have been encouraging; PAC epithelial cells have been detected growing on the lumen after two weeks' implantation in the nude mouse; (5) the _in vivo_ carcinogenesis investigations are still in progress. Pulonium

210 and nickel subsulfide have been implanted into the lumen of several xenotransplanted human subsegmented bronchi. Serial-sacrifice studies will begin within a few months.

Investigations in to the mechanism of asbestos carcinogenesis have shown: (1) amosite asbestos (100 - 1000 μg/ml) caused focal epithelial hyperplasia and atypical squamous metaplasia in human tracheobronchial explants; (2) amosite fibers were shown by both scanning and high voltage transmission electron microscopy to penetrate cultured epithelial cells. Short fibers (< 12μ) were found in the cytoplasm of the cells within 6 hrs, whereas longer fibers incompletely entered the cells. The epithelial cells did not show marked cell surface activity, and only small membrane sleeves around non-coated fibers were observed at the points of asbestos penetration; (3) to measure toxicity, asbestos (UICC samples; 0.1 to 100μ g/ml) was added to human bronchial epithelial cells that had been subcultured 24 hrs previously at clonal density. When compared to glass fibers, asbestos caused a statistically significant ($p < 0.05$) decrease in cell population doubling rate. Chrysotile was approximately 10-fold more cytotoxic than either amosite or crocidolite. Similar toxicity was observed using human bronchial fibroblastic cells.

Significance to Biomedical Research and to the Program of the Institute: The extrapolation of experimental animal data to man is a major problem in carcinogenesis. One approach to provide a link between these experimental data and human cancer is to develop model systems in cultured human tissues for carcinogenesis investigations. Such systems could be used for the identification of carcinogens and their metabolic pathways and, ultimately, in the identification of individuals who are highly susceptible to chemical carcinogens.

Proposed Course: Growth conditions for human epithelial cells will be continuously improved and the number of experiments designed to characterize the growth properties of these cells will be increased. Experiments studying the effect of tumor promoting agents are underway. Experiments are in progress to determine whether carcinogen-treated bronchial epithelial cells lose antigens commonly associated with normal cells and demonstrate the growth characteristics ascribed to transformed cells. Changes in these properties may be indicative of pre-malignant transformation. Investigations on single and multiple carcinogen exposures, as well as co-carcinogenesis with physical and viral agents on the development of neoplastic lesions in explant and cell cultures and xenotransplanted tissues will be continued and expanded. Ultimately, the mechanisms through which carcinogens transform epithelial cells will be investigated.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05133-02 LEP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Epidemiology Studies Using Monoclonal Antibodies to Carcinogen-DNA Adducts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Aage Haugen	Guest Worker	LEP	NCI
	John Groopman	Staff Fellow	LEP	NCI
	Curtis Harris	Assoc. Chief, LEP and Chief, HTSS	LEP	NCI
Others:	John Lechner	Expert	LEP	NCI
	Herman Autrup	Senior Staff Fellow	LEP	NCI

COOPERATING UNITS (if any)

Massachusetts Institute of Technology, Cambridge, MA; Cancer Institute, Beijing, Peoples Republic of China

LAB/BRANCH

Laboratory of Experimental Pathology (LEP)

SECTION

Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	1.6	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immunological approaches to measure DNA damage caused by carcinogens may be useful in biochemical epidemiology studies to identify individuals at high cancer risk. Mouse myeloma cells (P_3x63) were fused with spleen cells from Balb/c mice immunized with aflatoxin B_1-DNA adducts. Hybrid cells were grown in selective medium and tested for production of antibody-secreting hybridomas. Clones secreting monoclonal antibodies binding specifically to aflatoxin B_1-DNA adducts have been obtained. These antibodies have been characterized and, in conjunction with competitive ultrasensitive enzyme immunoassay, used to quantitate aflatoxin B_1 modified DNA in liver obtained from rats administered dosages ranging from 0.01-1.0 mg AFB_1/Kg. At this time, the limit of sensitivity is one aflatoxin B_1 residue per 1,355,000 nucleotides. Monoclonal antibodies to other carcinogen-DNA adducts are also being prepared.

Objectives: Monoclonal antibodies will be used to detect carcinogen-DNA adducts in human tissue specimens and cells and will be used in experiments such as 1) determination of exposure of individuals to chemical agents; 2) adduct-distribution in different organs; 3) DNA repair studies; and 4) experimental *in vitro* carcinogenesis.

Methods: Spleens from immunized mice are removed 2-3 days after the last immunization and minced in Dulbecco's phosphate buffered saline. Spleen cells (10^8) are mixed with myeloma cells (10^7), fused with polyethylene glycol and grown in selective medium. Myeloma cells will not grow in the selective hypoxanthine/aminopterin/thymidine (HAT) medium. Since spleen cells will not grow in culture, the only cells that survive are cell hybrids. Hybrid cells are dispersed in 96 well plates and incubated at 37°C. Cell growth after 14 days is taken as a successful hybrid. Cells can then be cloned with thymus cells added to the well as feeder cells. Supernatant from these clones is assayed with a modified enzyme-linked immunosorbent assay. Cells producing specific antibody are recloned and then expanded and injected i.p. into mice for development of ascites tumor. Monoclonal antibodies from ascites and cell culture medium are then isolated and characterized. The characterization procedures used competitive enzyme immunoassay and showed that the monoclonal antibodies recognized only aflatoxin bound to DNA and not free aflatoxin B_1-guanine adducts or six other aflatoxin B_1 metabolites. These antibodies were then used to quantitate aflatoxin B_1 metabolite bound to rat liver DNA following *in vivo* administration. These assays were performed using both ELISA and USERIA techniques.

Results: Immune response to aflatoxin B_1-DNA adducts were obtained by injecting methylated bovine serum albumin-AFB$_1$-DNA conjugate emulsified in Freund's complete adjuvant into mice. Hybridoma clones producing monoclonal antibodies against aflatoxin B_1-DNA adducts have been obtained and characterized. Competitive enzyme linked immunosorbent assay using these monoclonal antibodies reliably quantitated aflatoxin B_1 adducted *in vivo* to rat liver DNA at levels of one aflatoxin B_1 residue per 1,355,000 nucleotides. The competitive ultrasensitive enzyme radioimmunoassay was determined to be at least 10- to 12-fold more sensitive than the competitive enzyme linked immunosorbent assay in analysis of aflatoxin B_1 adducted DNA.

Significance to Biomedical Research and to the Program of the Institute:. Methods are being developed to quantitatively measure carcinogen-DNA adducts in femtomole and less amounts. These methods will be useful in studies of the molecular interactions of carcinogens and cell genome and in measurement of carcinogen-DNA adducts in biopsy specimens from people in high and low risk environments for cancer.

Proposed Course: Studies are under way to further characterize the antibodies and also to make monoclonal antibodies which specifically recognize other carcinogen-DNA products as well as isolated base adducts. These monoclonal antibodies and ultrasensitive enzymatic radioimmunoassay will be utilized to search for carcinogen-DNA adducts in human biopsy specimens and to determine the intra- and inter-genomic distribution of these adducts.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05134-02 LEP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Enzyme Immunoassay of Epidermal Growth Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Glennwood Trivers	Research Scientist	LEP	NCI
Other:	Curtis Harris	Assoc. Chief, LEP and Chief, HTSS	LEP	NCI
	John Lechner	Expert	LEP	NCI

COOPERATING UNITS (if any)

University of California, Irvine, CA; ICI, Ltd., United Kingdom

LAB/BRANCH

Laboratory of Experimental Pathology (LEP)

SECTION

Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.6	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Determining the sites of synthesis and the conditions affecting the secretion of growth factors, e.g., of mouse epidermal growth factor (mEGF), requires highly sensitive assays. We have, therefore, established conditions for the use of the ultrasensitive enzymatic radioimmunoassay (USERIA) to detect and quantitate such factors. Highly purified mEGF and anti-mEGF-IgG were reacted in polyvinyl microtiter plates, and the sensitivity of USERIA and the enzyme-linked immunoabsorbent assay (ELISA) were compared. The USERIA, a modification of the ELISA in which tritiated adenosine-5'-monophosphate (^{3}H-AMP) is substituted for p-nitrophenyl phosphate (pNPP), a colorimetric substrate, was shown to be superior to the ELISA in both noncompetitive and competitive assays. As little as 8 fmole mEGF has been successfully used as the solid-phase competitor for the USERIA. Experiments are now underway to determine the applicability of the competitive USERIA for measurement of growth factors in human cells.

Objectives: To employ the USERIA to study mEGF, human EGF (hEGF) and EFG-like substances in cells and tissues of both species before and after chemically induced malignant cell transformation.

Methods: DEAE purified mEGF, rabbit antisera and anti-mEGF-IgG; goat anti-rabbit-alkaline-phosphatase conjugate; modified enzyme-linked immunoabsorbent assay (ELISA) to optimize conditions for coating antigens to microtiter wells, reagent concentrations and reaction times, comparative ELISA and USERIA to optimize conditions for maximum sensitivity in the competitive (inhibition) USERIA; cell-free homogenates and conditioned media from cells cultured in 0% serum; USERIA (1) to study immunocompetition for anti-mEGF-IgG by molecules known (urogastrone) or thought to be related to mEGF, (2) to survey mouse and human tissues and cell cultures for the presence of associated EGF-like substances.

Major Findings: Assay procedures -- the optimum conditions for mEGF binding to polyvinyl U-bottom microtiter plates are aqueous buffered solutions, 37°C, and 16 to 20 hours incubation, beyond which the immunoreactivity rapidly decreases; drying mEGF in an aqueous unsupplemented solution for the same period causes significant loss of the immunoreactivity, and the remaining activity is not preservable in sealed plates at -20°C; plate related variability of coating efficiency requires standard curve controls for each experiment.

Assay characteristics -- in non-competitive reactions, substrate hydrolysis in both assays is proportional to increases in mEGF added to the plate; using 3×10^3 fmole anti-mEGF-IgG the ELISA achieves minimum significant detection (MSD) with 16 fmole in EGF within 16 to 24 hours incubation with pNPP; the USERIA achieves MSD with 8 fmole mEGF within 2-3 hours incubation with ^{3}H-AMP.

In competitive reactions, inhibition of substrate hydrolysis is proportional to increases in the amount of mEGF mixed with the antiserum of anti-mEGF-IgG in fluid-phase prior to being added to a plate with 32 fmole (or less) mEGF per well; using 3×10^3 fmole anti-mEGF-IgG/well in the fluid-phase, competition assays are routinely achievable in the USERIA with 16 fmole/well bound mEGF, and is also successfully performed with 8 fmole/well, within 2 to 3 hours incubation with the substrate; competition in the ELISA is routinely achievable with 32 fmole mEGF in approximately 3 to 4 hours, 16 fmole requires 16 to 24 hours, and 8 fmole, although detectable, gives A_{402} values in competition which are unacceptably low. This is also often true of the 16 fmole level. In competitive reactions with 16 fmole bound to the plate, the ELISA detects minimum significant inhibition (MSI) at approximately 8 fmole and 50% inhibition at 32 fmole in 18 hours; the USERIA detects MSI at 0.12 to 0.5 fmole and 50% inhibition at 2 to 8 fmole in 2 to 3 hours.

Experimental applications -- (a) purified preparations of the homologous hEGF (urogastrone) have been obtained, and preliminary results indicate that this molecule, which differs structrually from mEGF by 13/53 sequential amino acid residues, will compete for the anti-mEGF-IgG under the optimun conditions used for mEGF, causing 50% inhibition at 8 fmole, similar to that acheived with mEGF; (b) preliminary results using cell-free homogenates and conditioned medium from cultures of human tumor cell line indicate that both contain a

Significance to Biomedical Research and the Program of the Institute: The isolation of homologous epidermal growth factors from the tissues of mice (mEGF) and humans (hEGF, urogastrone) strongly suggest an important role for these polypeptides in mammalian biology. Moreover, the recent discovery and accumulation of growth factors secreted by transformed cells imposes a high priority on the need to illucidate the mechanism of their production. Development of ultrasensitive assays for the study of these factors is essential to the delineation of their role in influencing normal and abnormal cellular proliferation and differentiation, particularly their potential in diseases such as cancer. Currently, the one sensitive assay published for mEGF is an RIA employing I^{125}-labeled mEGF. It detects MSD at 30 pg (4.69 fmole) in competition; the current level of refinement in the USERIA for mEGF represents a 38-fold increase in the sensitivity. This significant increase in the ability to detect mEGF allows the measure of mEGF or mEGF-like factors associated with relatively small numbers of cells, perhaps in the primary foci of altered *in vitro* populations. Compared to existing methods, the assay is more sensitive, less expensive, faster and safer to conduct. An extremely important potential now exists in the possibility that the mouse anti-mEGF-IgG may be effectively used to study production of hEGF.

Proposed Course: Continuation of effort to optimize conditions for competitive USERIA; competitive measure of known amounts of mEGF added to sera, urine, clarified cellular lysates and culture medium and the quantitative study of the *de novo* levels of EGF-like substances from these sources; confirmation and refinement of procedures for USERIA measurement of hEGF; development of protocols for the study of EGF production before and after chemically induced and/or malignant transformation in mouse and human cells *in vitro*.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05157 02 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Biochemical Factors that Contribute to Organ-specific Carcinogenesis and Selectively Modify the Susceptibility of Fetal Tissues to Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.M. Rice	Section Chief	LEP NCI
Other:	B. Chen	Research Chemist	LEP NCI
	P. Donovan	Chemist	LEP NCI
	A. Perantoni	Microbiologist	LEP NCI

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Experimental Pathology

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Factors that modify tissue responses to chemical carcinogens in different organ systems are studied to evaluate their contribution to changing susceptibilities to carcinogenesis in certain tissues during prenatal and postnatal development. Tissues and organ systems currently under study include the nervous system, kidneys, liver, and intestinal mucosa. Modifying processes now being studied include excision repair of DNA, transport of carcinogen into and within cells, and the changing cellular susceptibility to carcinogens during different stages of the cell cycle. Factors investigated in the past, and anticipated for future study, include immune responses to tumors, the induction and genetics of enzymes metabolizing carcinogens, and tumor promotion.

Objectives: To define the roles of biochemical processes that significantly modify the susceptibility of fetal and selected adult organs to chemical carcinogens. Current specific objectives include the following: to investigate the development of capacity for excision repair of DNA in fetal tissues, including brain and liver, during the course of intrauterine development and to evaluate the role of repair capacity or its absence in high prenatal susceptibility to tumorigenesis in these organ systems in different species; to evaluate the role of the enzyme gamma glutamyltranspeptidase (GGT) in the pronounced organ selectivity of the carcinogen azaserine and related substances which are selectively toxic and carcinogenic for organs such as the kidney and pancreas that are characteristically high in levels of GGT; to evaluate the extent to which susceptibility of a specific target cell, the hepatocyte, varies in susceptibility to chemical carcinogens during different stages of the cell cycle, a factor of major importance in understanding the intrinsically high susceptibility to chemical carcinogens of fetal tissues that have high rates of cell division.

Methods Employed: For studies of active transport phenomena involving GGT, cells possessing this enzyme and derivative, or mutant lines which do not, are grown in cell culture, and the effects of toxic substrates of the enzymes are evaluated morphologically. Uptake and distribution of toxic compounds is determined by liquid scintillation counting of radioactive preparations. Enzyme activities are demonstrated histochemically and quantitated biochemically through standard procedures involving the generation of colored enzyme reaction products. Nucleic acid repair in organs taken directly from fetal tissues is accomplished through alkaline elution procedures without radiolabeling of the target tissues and by direct measurement of enzyme activities such as apurinic endonuclease that are required for DNA repair. Nucleic acids are quantitated by different fluorescence emission spectra of bound and non-bound ethidium bromide. Pregnant animals are treated with various doses of carcinogens at precisely defined times during gestation; and at selected intervals after treatment, fetal organs are dissected under a microscope and dissociated for study by the elution system. The cell cycle kinetics of the regenerating rat liver are determined after subjecting F344 rats to a two-thirds partial hepatectomy, by pulse labeling the regenerating liver with thymidine, quantitating the incorporation of this DNA precursor by liquid scintillation counting of isolated DNA, and by evaluating the proportions of cells undergoing DNA synthesis and in mitosis through high resolution autoradiographic techniques. Rats are injected via the portal vein with selected doses of direct-acting carcinogens at precisely defined times after partial hepatectomy and the carcinogenic response, as well as the evolution of preneoplastic lesions, is determined as a function of time elapsed since partial hepatectomy and as a function of the stage of the cell cycle at which carcinogen treatment took place. Increased synchrony in regenerating liver is achieved by post-hepatectomy injection with hydrocortisone hemisuccinate at 5 hour intervals for a total of 3 injections.

Major Findings: Previous reports have documented that for a number of cell lines, sensitivity to toxicity by the carcinogen L-azaserine correlates positively with intracellular levels of GGT.

Studies of possible mechanisms for the observed correlation between GGT activity and sensitivity to azaserine-induced toxicity have continued. Despite reports that GGT might function in an active transport process for neutral amino acids, we have conclusively determined that GGT does not play such a role in the human nephroblastoma line TuWi. Azaserine selection results in the preferential survival of cells deficient in GGT. When these cloned strains are analyzed for uptake of azaserine or other neutral amino acids, uptake is not reduced in strains deficient in GGT. In addition, the potent GGT-inhibitor complex, serine-borate, is unable to decrease azaserine uptake in cultures of TuWi. We proposed an alternative mechanism to that of uptake based upon reports by others studying the azaserine analog diazo-oxonorleucine (DON). E. coli glutaminase was found to actively metabolize DON to its highly reactive diazomethane form. Since GGT has glutaminase activity, perhaps it could activate azaserine. Azaserine was found to be a good substance for the E. coli enzyme; however, comparable activity was not demonstrated using GGT purified from rat kidney. The bacterial enzyme may be responsible for the extensive intestinal mucosal toxicity associated with azaserine exposure. Another possible mechanism under study involves reported regulation by GGT of intracellular glutathione levels. We have found that azaserine will react with reduced glutathione and that reduced glutathione and cysteine, but not oxidized glutathione, protect TuWi cells from azaserine toxicity. In addition, diethyl maleate, which consumes intracellular glutathione, enhances cellular sensitivity to azaserine. We are presently investigating cellular glutathione levels to determine if these levels correlate with sensitivity to azaserine. If our GGT-deficient TuWi strains, which are azaserine resistant, contain elevated glutathione levels, they would be extremely useful for studying the importance of thiols in the activation or detoxification of alkylating species.

Elevated GGT activities are often associated with proliferative cell populations and consequently may provide cells with a growth advantage. To test this hypothesis, cells from TuWi strains which contain divergent GGT activities were injected into athymic mice. A positive correlation was observed between the ability of a strain to survive and grow in vivo and its level of GGT activity. This finding is consistent with our hypothesis that GGT function confers preferential growth capacity.

Quantitative excision repair studies continue in order to explore the relationship between DNA repair activity and organotropic differences in fetal susceptibility to chemical carcinogenesis. Pregnant rats received a single dose of ethylnitrosourea during late gestation. Cell suspensions from fetal brain, kidney, and liver were subjected to DNA fragmentation analysis as a function of time after exposure to the carcinogen. Repair of alkylation sites resulting from exposure to ENU involves sequential formation of apurinic or apyrimidinic sites in DNA, followed by single-strand breakage and rejoining of the break, or alternatively, enzymic cleavage of the DNA strand in which damage has occurred at some point of variable distance from the break, removal of DNA containing the damage, resynthesis and rejoining of the two resulting breaks.

such agents. The factors that contribute to this enhanced susceptibility remain to be adequately evaluated. The role of DNA repair processes, best shown by enhanced susceptibility to UV carcinogenesis in individuals deficient in such repair as a result of the hereditary condition xeroderma pigmentosum, indicates that repair is significant in controlling the consequences of damage to cells inflicted by at least some carcinogenic agents, but the development of this capacity during prenatal life has been studied very little. It could easily contribute, in part, to the susceptibility of fetal tissues which may be deficient in activity or fidelity of DNA repair.

Organ specificity in carcinogenesis by different classes of agents is of obvious importance to the problem of extrapolating between species and in understanding the spectrum of tumors ascribed to environmental causes in human beings. Most studies in this area have concentrated on capacity of target cells to metabolize carcinogens to ultimate reactive forms. Other possible mechanisms have received little attention in comparison and deserve exploration. The role of target cell constituents, such as GGT as possible determinants of organ specific carcinogenesis, is a step in this direction.

It has long been known that dividing cells are more intrinsically susceptible to chemical carcinogens than post-mitotic cells or cells that are not cycling. The exact reasons for this are not clear; a reasonable hypothesis is that DNA may be more vulnerable to damage in certain phases of the cell cycle, and that in cycling cells, there is a greater probability that DNA repair will not be completed before programmed DNA synthesis encounters a defective portion of the genome bearing a carcinogen-induced lesion. Whether, in fact, cells are especially vulnerable to carcinogens at a particular stage of the cell cycle is thus of importance not only for perinatal carcinogenesis, but for carcinogenesis in adult tissues where mitotic activity is high, including various lining epithelia such as intestinal mucosa which constitute major sites of important neoplasms of man.

Proposed Course: The prenatal development of capacity for excision repair of DNA in liver, brain, and other tissues will continue to be studied by high-resolution alkaline elution and by assays for activity of repair enzymes, in a continuing effort to titrate physical repair of single-strand breaks at dosages of simple monofunctional methylating and ethylating agents below cytotoxic levels, as judged from histological sections of organs of interest. This procedure should allow for greater resolution than the relatively crude alkaline sucrose gradients initially employed, will eliminate artifacts due to radioactive decay of tritium labels and the severe selection process of short term *in vitro* cultivation for purposes of labeling, and should allow meaningful comparison of species of experimental animals that differ greatly in prenatal susceptibility to carcinogens in various organ systems. Carcinogens with more complex chemical structures will be subsequently studied in the same fashion to determine which types of damage are repairable by fetal tissues, and how these correlate with prenatal susceptibility to carcinogenesis in different organ systems in different species.

Studies on the role of the cycle in susceptibility to carcinogenesis, and possibly other forms of genotoxic damage including mutation, will be continued.

The mechanism of DNA repair deficiency in fetal rat brain versus other organs was further studied by comparing the activities of a key repair enzyme, apurinic/apyrimidinic (AP) endonuclease, in tissue extracts. A standard filter binding assay, using the circular replicated form (RF) of bacteriophage ΦIX174 which was biosynthesized and used as the substrate, did not clearly show any difference among extracts from different fetal organs. When DNA which contains genome coding for enzyme β-galactosidase was used as template, extracts from fetal rat liver regularly revealed a high level of AP endonuclease. This second method assays the template activity of target DNA to direct enzyme synthesis _in vitro_.

The high level of AP endonuclease activity in rat liver resides in the nuclear fraction, but not in the 100,000xg supernatant (S100) fraction. An intermediate level of nuclear AP endonuclease was found in kidney, an organ of intermediate susceptibility to carcinogenesis. Very little activity was observed in any fraction of the extracts from fetal rat brain, a tissue very highly susceptible to prenatal carcinogenesis. These studies are continuing in order to place these results in the context of alkaline sucrose gradient studies and O^6-alkylguanine excision studies previously reported from this laboratory and from other laboratories which suggest a significant deficit in nucleic acid excision repair capacity in the rat brain, especially during late fetal life when susceptibility to chemical carcinogens is maximal.

Studies on the effects of chemical carcinogens on the regenerating rat liver indicate that in synchronized hepatocyte populations in regenerating livers, susceptibility to neoplastic transformation by the alkylating agent methyl-(acetoxymethyl) nitrosamine (DMN-OAc) is greatest in cells that are actively synthesizing DNA, and thus that the S phase of the cell cycle, especially its earlier portion, is a period during which there is markedly enhanced intrinsic cellular vulnerability to neoplastic transformation. The conclusions are especially convincing since the onset of S could be delayed by as much as 10 hours by administering the hormone hydrocortisone by intraperitoneal injection at intervals after partial hepatectomy, resulting not only in a delay in onset of S, but improved synchrony once decay of the block allowed the cell cycle to proceed in the regenerating liver. Delay of 10 hours in the onset of S delayed the peak tumor incidence in carcinogen-treated rats by the same interval. DMN-OAc, an ester of the presumed reactive metabolite of dimethylnitrosamine, which is dependent on esterase for its activation rather than any oxidative enzyme system, resulted in a high incidence of hepatocellular tumors, nodules, and foci, and caused practically no tumors in other organ systems when injected into partially hepatectomized rats via the portal vein. Our experiments indicate that this is the ideal agent to use for cell cycle studies since levels of esterases do not change over the period of hepatectomy and regeneration in liver and labeling of DNA by DMN-OAc-C^{14} is essentially constant, irrespective of the stage of the cell cycle in regenerating liver when the labeled carcinogen was given.

Significance to Biomedical Research and the Program of the Institute: The biochemical basis of the well-established high fetal susceptibility to chemical carcinogens remains poorly understood. The fetus is clearly at greatly elevated risk from exposure to chemical carcinogens, a fact that must be considered in estimations of relative human risk from environmental exposure to

Techniques developed in the course of these studies, including characterization of the biology and morphology of liver cell tumors in rats resulting from a single transient exposure to an alkylating agent, will be applied elsewhere in the program of this Section. We plan to attempt to resolve apparent discrepancies between capacities for DNA repair in fetal liver and brain in rats and mice, and the distinct differences in susceptibility to oncogenesis in these species. Prenatal susceptibilities to hepatocarcinogenesis are at variance with studies of repair in the fetal and postnatal nervous system and liver in both species. One approach to this problem will be through studies of tumor promotion in the liver. It is well known that agents such as phenobarbital are capable of acting as classic tumor promoters on hepatocytes in both the rat and the mouse. Experiments will be conducted to determine whether postnatal promotion by phenobarbital will promote expression of latent neoplastic hepatocytes in the livers of rats transplacentally exposed to alkylating agents such as ENU, which in the absence of such postnatal promotion would never proliferate to form tumors. In addition, the fact that liver cell tumors are inducible not only in the mouse, but in sub-human primates by prenatal exposure to alkylating agents, invites the utilization of phenobarbital in transplacental carcinogenesis studies in primates to demonstrate promotion phenomena in a primate species in an experimental context for the first time. This would be a major contribution to the available data base on which extrapolation between species of the effects of tumor promoters could be based, which at the present time is restricted entirely to rodents and therefore only hypothetical as a significant category of human risk.

Finally, the role of fetal metabolism versus maternal metabolism as a determining factor in the capacity of metabolism-dependent carcinogens to act transplacentally has, in the past, been studied in this Section indirectly through studies of the biological effects of agents whose metabolic paths are known. The recent literature on metabolism of foreign compounds, however, suggests that conclusions on the role of fetal metabolism based entirely on responses to polynuclear aromatic hydrocarbons may not be applicable, and in fact, may run counter to the effects of other important classes of carcinogens including the nitrosamines. Direct study of this phenomenon in both biochemical and genetic contexts is planned as a future activity of this Section and is dependent on recruitment of a scientist experienced in techniques for studying carcinogen metabolism in the laboratory to participate in these experiments.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05177-01 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Use of Immunological Techniques to Study the Interaction of Carcinogens with DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.C. Poirier	Chemist	LEP	NCI
Other:	S.H. Yuspa	Chief, In Vitro Pathogenesis Section	LEP	NCI

COOPERATING UNITS (if any)
Human Tissues Studies Section
Dr. John Stanley - NIDH
Dr. I.B. Weinstein -Columbia University
Dr. Brian Laishes -McArdle Laboratories
Dr. David Kaufman -University of North Carolina

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
In Vitro Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.25	1.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies have employed antibodies to carcinogen-DNA adducts to investigate the extent and nature of binding of 2-acetylaminofluorene (2-AAF) and benzo(a) pyrene (BP) to DNA in vivo and in vitro. By radioimmunoassay it is possible to quantitate and distinguish between the acetylated and deacetylated C-8 adducts of 2-AAF. In vitro studies with this assay have concentrated on determining and manipulating the adducts formed in the DNA of cultured cells upon exposure to N-acetoxy-2-acetylaminofluorene. The nature and extent of binding has also been monitored in the livers and kidneys of male rats fed a carcinogenic dose of 2-AAF for up to 16 weeks. Antisera raised against BP bound to deoxyguanine in DNA have been utilized in ELISA and USERIA assays to detect less than 1 femtomole of BPdG adducts, and human tissues from individuals exposed environmentally or occupationally to polycyclic aromatic hydrocarbons are being screened for the presence of adducts on DNA. The BP-DNA antisera have also been used for the visualization of BP adducts on calf thymus DNA by electron microscopy and in nuclei of BP-exposed cultured cells by immunofluorescence.

Objectives: To develop and utilize specific and sensitive immunological methods to monitor the interaction of carcinogens with DNA. Studies are directed toward quantification of the extent of in vivo covalent binding and removal under experimental conditions, and toward determining the structural and functional consequences of the formation of specific carcinogen-DNA adducts.

Methods Employed: Both in vivo carcinogen exposure to experimental animals and carcinogen treatment of cultured cells are employed to pursue the objectives. Tissues and cells obtained from patients environmentally exposed to carcinogens are also utilized. The chemical synthesis of radiolabeled and unlabeled DNA-carcinogen adducts and their purification by column chromatography are currently performed. Isolation of macromolecules for carcinogen binding and repair studies utilize density gradient centrifugation. Antibodies are produced by injection of purified antigens into rabbits and by monoclonal technology. A variety of immunological techniques are employed including the qualitative procedures of immunofluorescence and immunochemical electron microscopy and the quantitative radioimmunoassay (RIA) and enzyme-linked radioimmunoassay (ELISA). High performance liquid chromatography (HPLC) is being established for characterization of specific adducts.

Major Findings: The interaction of carcinogens with DNA has been studied by a unique methodology pioneered by this Section. Antibodies have been developed in rabbits against guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin-(8-yl)-2-aminofluorene (G-8-AF), the major guanosine adducts formed in vivo and in vitro by the interaction of nucleic acids with the aromatic amine carcinogen 2-acetylaminofluorene (AAF). The antisera obtained have been employed to detect picomoles of each C-8 adduct in a competition radioimmunoassay utilizing ^{3}H-G-8-AAF or ^{3}H-G-8-AF. A linear regression equation has been established which allows precise quantitation of each C-8 adduct in an unknown mixture in which dG-8-AAF comprises $\leq$ 10% of total C-8 adduct; for mixtures with more than 10% dG-8-AAF, proportions can be estimated by comparison with standard mixtures.

A variety of cultured cells from different species have been exposed to N-acetoxy-acetylaminofluorene (N-Ac-AAF) and adduct formation has been surveyed to determine the innate capacity of each cell type to deacetylate the carcinogen during the process of adduct formation. The deacetylated adduct is more mutagenic in bioassays. The validity of the RIA approach in performing such studies was confirmed by concomitant HPLC analysis of DNA-binding profiles. The data indicate that specific patterns of AAF-DNA binding are determined at the cellular level and that experimental techniques can be developed to modify binding profiles. Studies are currently in progress to assess the effect of binding patterns on carcinogenesis and mutagenesis.

Immunological techniques have also been applied to study AAF binding to liver DNA during liver carcinogenesis protocols. In collaboration with Dr. Brian Laishes at the McArdle Laboratory for Cancer Research, RIA has been used to detect C-8 adducts in liver and kidney DNA of male Wistar-Furth rats fed 0.02% or 0.04% 2-AAF either continuously for up to 16 weeks or for a specific time followed by a carcinogen-free interval for repair. With dietary AAF, substantial levels of binding (80 fmoles/μg DNA) were already observed in the liver DNA after 24 hours. The binding values increased for both 0.02% and 0.04% AAF fed continuously, reaching a plateau of approximately 230 fmoles/μg DNA at 30 days and thereafter. During the first week of continuous feeding, about 80% of the total C-8 adducts in the liver DNA were deacetylated (dG-8-AF) and the proportion of dG-8-AF increased to 97-100% by 15 and 30 days of feeding for the 0.04% and 0.02% 2-AAF diets, respectively. In separate experiments, rats were fed 2-AAF for 3, 7 or 28 days, the carcinogen diet was discontinued and the liver adducts assayed at 1, 7 and 28 days after removal of the 2-AAF. When dietary 2-AAF administration was for 3 or 7 days, approximately 70% of the C-8 adducts remained on the liver DNA 7 days after a return to control diet, and 7-35% remained at 28 days. However, when dietary administration of 2-AAF was for 28 days, most of the C-8 adducts were still present after a 28 day repair interval. In all experiments, the formation of C-8 adducts in kidney DNA averaged 10-15% of the liver values and followed the same binding and repair profiles as liver. These results demonstrate that removal of specific adducts may be preferential during liver carcinogenesis and adducts may persist for a prolonged time period after withdrawal of carcinogen exposure.

Antisera have also been developed against DNA substituted with the 7,8-diol, 9,10 epoxide of benzo(a)pyrene (BPDE I, the anti-isomer). The antigen was synthesized to yield > 99.9% of adducts as trans(7R)-(benzo(a)pyrene)-N^2-deoxyguanosine (BPdG). The antisera have a higher affinity for BP substituted DNA than for the isolated BPdG adduct, suggesting antibody recognition of the DNA backbone. The antisera do not cross-react with deoxyguanosine, DNA or the carcinogen alone. The ability of this antibody to recognize BPdG in intact (non hydrolyzed) DNA makes it particularly useful for morphological detection. Studies conducted during the past year, in collaboration with Dr. David Kaufman of the University of North Carolina, have indicated that visualization of adduct on DNA is possible by ultrastructural techniques. Antiserum was incubated with calf thymus DNA, modified in vitro to various extents with BPDE-I, subsequently incubated with ferritin conjugated goat anti-rabbit IgG, and examined by electron microscopy using a modification of the Kleinschmidt basic protein monofilm technique. DNA modified by BPDE-I to 0.005% and less resulted in EM visualization of over 60% of the BPDE-I-DNA adducts identified by radioimmunoassay. However, more highly modified DNAs gave less quantitative indications of extents of modification in this system due to aggregations of DNA and antibodies. No specific binding of antibodies was visualized with unmodified DNA or when pre-immune serum was incubated with unmodified or BPDE I-modified DNA. This system has been used to examine BPDE-I modification of DNA from nuclei of regenerating rat liver and results are currently being evaluated.

Qualitative studies were also performed at the cellular level by examining cultured mouse epidermal cells exposed to BPDE-I in vitro by indirect immunofluorescence. Bright nuclear fluorescence was observed in BPDE-I-exposed cells with specific antiserum, but normal serum absorbed specific serum, and non-exposed cells did not yield fluorescence. Specific fluorescence was abolished by treatment of fixed, permeabilized cells with DNAase. RNAase treatment removed particularly intense staining associated with nucleoli but did not change overall nuclear fluorescence. While binding was readily seen one hour after BPDE-I exposure, by 24 hours it could no longer be observed suggesting removal of adduct during that time to levels below detectability by this technique. These studies have been carried out in collaboration with Dr. John Stanley of the Dental Institute at NIH.

The quantitative analysis of BPdG formation was enhanced by the development of ELISA and USERIA assays in collaboration with the Human Tissue Studies Section. These enzymatic amplification assays have yielded sensitivity in the range of less than 1 femtomole and allow detection of adduct from biological samples exposed to small quantities of B(a)P. Using the ELISA assay, samples of human lung and white blood cells from individuals exposed to B(a)P through lifestyle (smoking) or occupation (coke oven or shale retort workers) are being monitored for adducts. The collection of the clinical material is currently being coordinated by Dr. I.B. Weinstein of Columbia University.

Significance to Biomedical Research and the Program of the Institute: The development of immunological procedures for the investigation of carcinogen-DNA interactions has provided a powerful tool for the study of this phenomenon. Experimentally antibodies are more specific and sensitive and less costly than conventional radiolabeled probes used for such studies. Standard carcinogenesis protocols need not be modified for DNA-binding studies and prolonged sequential administration can be monitored. Morphological approaches can be employed to determine inter- and intracellular distribution of adducts, and our most recent results indicate even intramolecular localization is possible. Antibodies may also be useful to probe for adducts in humans exposed to carcinogens, both to monitor a biological effect of a specific exposure and to gather data on dose-response in prospective epidemiological studies.

Proposed Course: The remarkable sensitivity and specificity of antibodies raised against carcinogen-DNA adducts provides a new approach to bioassay and mechanistic studies. As an applied methodology, this technique may allow for detection of persistent adducts in cells and tissues from human populations exposed to environmental carcinogens such as benzo(a)pyrene. During the past year, we have succeeded in identifying conditions to alter the ratio of acetylated and deacetylated adducts obtained after exposure of epidermal cells to N-Ac-AAF. We are currently examining the effect of varying the relative ratio of each of these C-8 adducts in carcinogenesis and mutagenesis assays. In vivo studies will be directed toward the monitoring of AAF adducts in normal and altered liver foci from rats fed AAF and investigating the apparent loss in repair capacity observed after 28 days on the 2-AAF diet. Antibodies to the dG-N^2-AAF derivative, the BP-polyadenosine adduct, AAF-poly dG (which yields a Z DNA structure) and cis-platinum-DNA adduct will be elicited by conventional rabbit injection or by monoclonal techniques. The monoclonal antibodies are being produced in collaboraration with Dr. Ray Gilden at the Frederick Cancer Research Center. Collaborative studies utilizing electron microscopic immunohistochemistry (ferritin labeling) to detect specific localization of BP adducts on DNA will focus on DNA exposed in vivo and on a search for selective binding sites in fractionated DNA. In particular, binding to initiation sites in replicons is being examined after isolation of these sites by density gradient centrifugation. We will also use this technique to determine if gaps in nascent DNA after exposure of cells to BPDE-I are localized opposite adducts. Immunofluorescence techniques will be applied to study tissue distribution of bound carcinogen. In collaboration with other members of the Section, antibody will be used to localize binding in particular DNA sequences (cloned genes) and to determine the functional consequences of the adduct on gene regulation.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05178-01 LEP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	James Strickland	Research Chemist	LEP	NCI
Other	Stuart H. Yuspa	Chief, In Vitro Pathologist Section	LEP	NCI
	Henry Hennings	Senior Chemist	LEP	NCI

COOPERATING UNITS (if any)

Experimental Ontogeny Section
Lab. Cellular & Mol. Biol.
NCI-FCRC

Somatic Studies Section
Div. of Biological Effects
Bureau of Radiological Health
FDA

Mol. Carcinogenesis Group
Cancer Toxicology Section
Biology Division
Oak Ridge Natl Lab

Differentiation Control Section,
Lab. Exptl Pathol., NCI

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
In Vitro Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SENCAR mice are markedly susceptible to two-stage skin carcinogenesis compared to BALB/c mice. Susceptibility is not due to differences in metabolism of polycyclic aromatic hydrocarbons between SENCAR and more resistant strains. Sensitivity appears to be determined by the target tissue since SENCAR, but not BALB/c, skin grafted to nude mice developed papillomas at a high frequency after initiation and promotion. DNA repair capacity, studied by host cell reactivation, utilizing ultraviolet light-irradiated herpes simplex virus, was similar in cultured epidermal cells of BALB/c and SENCAR. SENCAR cells have a greater binding capacity for epidermal growth factor than BALB/c cells, but the increased binding in response to retinoic acid and the rapid decrease after phorbol ester exposure are similar in the two strains. Expression of endogenous xenotropic type C proviral sequences occurs more readily in BALB/c. The frequency of spontaneous and carcinogen-induced, differentiation-resistant foci in vitro is greater in SENCAR than in BALB/c epidermal cells. These results suggest that susceptibility for skin carcinogenesis in SENCAR mice is a property of the skin itself and has no clear relation to DNA excision repair, endogenous virus expression, or EGF receptors.

Objectives: To elucidate the cellular mechanism of genetically derived, enhanced sensitivity to carcinogenesis.

Methods Employed: The SENCAR mouse is extremely sensitive to chemically induced skin cancer. This strain was developed by a selective breeding protocol for increased susceptibility to skin tumors produced by DMBA initiation and croton oil promotion. In order to elucidate the basis for this susceptibility, SENCAR mice are exposed to carcinogens and tumor promoters *in vivo* or SENCAR epidermal cells are cultured and studied *in vitro*. Grafting of SENCAR skin to nude mice to separate host and target tissue properties is also performed. Similar studies are performed on resistant mouse strains to investigate the contributions of DNA repair, endogenous viruses, growth factors, immunological mechanisms (Langerhans cells), and other factors to the increased sensitivity of SENCAR mice. DNA repair capabilities are assessed *in vitro* by the ability of cultured cells to functionally repair (host cell reactivation) ultraviolet (UV) light- or carcinogen-damaged herpes simplex virus after infection of primary epidermal cells with damaged virus. Activity of DNA polymerase, the major repair polymerase is determined by *in vitro* polymerization assay and has been compared in epidermal cell lysates from cultured cells derived from sensitive and resistant animals. Receptor binding studies with cells in culture have been performed using ^{125}I-labeled epidermal growth factor (EGF) and ^{125}I-labeled virus membrane glycoprotein gp70. Target cells specific for xenotropic and ecotropic type C RNA viruses have been utilized to propagate infectious viruses in epidermal cells, homogenates, and culture fluids. Reverse transcriptase assay of culture fluid from target cells was done to detect replicating viruses. Epidermal Langerhans cells have been identified in primary cultures by rosette formation with sensitized red blood cells and by ATPase staining. Cultured epidermal cells are assayed for resistance to terminal differentiation by culture under conditions of reduced or increased extracellular calcium.

Major Findings: Recent studies indicated that the increased susceptibility of SENCAR mice to skin carcinogenesis relative to other strains, is not accounted for by differences in metabolism of polycyclic aromatic hydrocarbon carcinogens. To determine whether the sensitivity to carcinogenesis is a property of the skin itself or is dependent upon host factors, skin was grafted from SENCAR and BALB/c (a resistant strain) to the backs of athymic nude mice, and the grafts were subjected to an initiation-promotion protocol. Tumors arose only in SENCAR skin grafts, and with high frequency implying that the increased susceptibility was a property of the skin itself. SENCAR mice were also susceptible to initiation by N-methyl-N'-nitro-N'-nitrosoguanidine (MNNG) a carcinogenic nitrosamide which does not require metabolic activation. Newborn SENCAR are also highly susceptible to skin carcinogenesis suggesting that the susceptibility is not acquired during maturation. SENCAR, but not BALB/c, skin develops significant numbers of tumors with promoter alone in the absence of initiation. This suggests that initiated cells may preexist constitutively in SENCAR mice. The preexistence of initiated cells is supported by *in vitro* studies in which colonies of epidermal cells resistant to Ca^{++}-induced terminal differentiation developed in primary cultures of SENCAR, but not BALB/c skin. These colonies were similar to colonies from other strains which were produced by carcinogen treatment of basal cells and subsequent selection by Ca^{++}-induced differentiation.

Preliminary studies utilizing specific antibodies to carcinogen-DNA adducts had suggested that SENCAR cells might be deficient in removal of adducts in comparison with BALB/c cells. This suggested the possibility that SENCAR cells had a defect in DNA repair. This was explored further by several techniques. Primary epidermal cells from SENCAR, CD-1, BALB/c, and AKR mouse strains, which form a spectrum of sensitivities to skin carcinogenesis, were equally capable of reactivation of UV-inactivated herpes virus. Comparison of the activity of the repair enzyme DNA polymerase in BALB/c and SENCAR cells demonstrated similar levels of enzyme. No significant changes in polymerase β activity occurred after treatment of cells from either strain with the potent skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Binding of acetylaminofluorene to SENCAR and BALB/c epidermal cells exposed in vitro was quantitatively and qualitatively similar. These results indicate that susceptibility to carcinogenesis in SENCAR cells cannot be accounted for on the basis of alterations in DNA binding or repair.

Evidence for spontaneous expression of endogenous xenotropic, but not ecotropic, type C RNA virus was found in both BALB/c and SENCAR epidermal cells in culture. This expression occurred at low levels, however, since infectious virus was demonstrable only by cocultivation of epidermal cells with appropriate target cells and not in homogenates or culture fluid from these same cells. Furthermore, virus expression was seen in SENCAR only in a cell line with altered differentiation properties derived from primary epidermal cells by treatment with the carcinogen MNNG followed by exposure to TPA, whereas virus was detected in primary BALB/c epidermal cells as well as lines with altered differentiation properties derived as above. Epidermal cells from BALB/c and SENCAR had similar levels of surface receptors of gp70, the major murine leukemia virus (MuLV) envelope glycoprotein, and were good target cells for infection with Rauscher and AKR MuLV. The latter result implies that transformation by infection with MuLV pseudotypes of murine sarcoma virus can be done. Taken together, these data do not suggest a direct role of infectious virus in the susceptibility of SENCAR mice but cannot rule out a role for integrated viral sequences.

Recent studies have suggested that response to endogenously secreted growth factors may be associated with maintaining the malignant phenotype. SENCAR cells and cells derived from less sensitive strains were compared for growth factor receptor properties.

SENCAR primary epidermal cells had 1.2 - 2.0X the level of epidermal growth factor (EGF) binding seen in BALB/c cells, but there was no quantitative correlation with sensitivity to carcinogenesis when CD-1 and AKR strains were included in the comparison. EGF binding in either SENCAR or BALB/c cells was not greatly changed in epidermal cell lines when compared to primary cells even though these lines had altered differentiation properties and in some cases were tumorigenic. TPA treatment of BALB/c or SENCAR cells caused a rapid and pronounced reduction in EGF binding of 90% or more. Retinoids (retinoic acid and retinol) caused slight increases (less than 2X) in EGF binding. This effect was somewhat more pronounced for BALB/c than for SENCAR cells. Induction of terminal differentiation by increasing Ca^{2+} concentrations in the media led to decreased (30-70%) binding of EGF by cells of both strains. In this case, SENCAR cells appeared to be affected to a greater extent than BALB/c cells. It is unlikely that these minor differences in EGF receptor biology could account for the susceptibility of SENCAR but more significant differences in receptors for other growth factors cannot be ruled out.

There appear to be significant differences between BALB/c and SENCAR skin in the abundance of epidermal Langerhans cells. These suprabasal cells, which are in the macrophage family, may be responsible for certain aspects of antigenic responses in skin. They are detected by their ability to form rosettes with sheep red blood cell-IgG complexes due to the presence on the surface of Langerhans cells of receptors for the F_c fragment of IgG. Langerhans cells also stain for ATPase under properly controlled conditions. BALB/c epidermis appears to contain as much as 8X as many Langerhans cells as SENCAR. Langerhans cells comprise about 4% of BALB/c epidermis while SENCAR has about 0.5% Langerhans cells. The reduced Langerhans cell population in SENCAR is not a result of overall macrophage deficiency. Langerhans cells attach to the plastic substrate in culture and become strongly resistant to removal by treatment with trypsin. Thus, one can select conditions which will favor detachment of keratinocytes and leave a sparse culture of nearly 100% Langerhans cells. These cells appear to replicate in vitro. Langerhans cell deficiency in SENCAR mice could lead to a greater sensitivity to carcinogens as a result of a failure in an immune surveillance mechanism in which Langerhans cells identify latent tumor cells for destruction by natural killer cells. Such a mechanism would not explain the altered response to terminal differentiation observed in SENCAR keratinocytes in vitro.

Significance to Biomedical Research and the Program of the Institute: Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases, specific genetic changes have been associated with increased risk, but in many examples, polygenic influences appear more likely. To date biochemical epidemiological studies have focused only on genetic differences in carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact it seems likely that factors associated with the expression of neoplastic change would play an important role in host susceptibility. The development of animal strains through selective breeding with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. In vivo studies have indicated that carcinogen metabolism is unlikely to explain the sensitivity of SENCAR and grafting experiments indicate the target tissue itself is somehow more susceptible. This validates the use of in vitro techniques to explore susceptibility. In vitro studies have suggested that certain biological alterations associated with carcinogen exposure are constitutive in the susceptible strains and that immunological differences could also be involved in the expression of neoplastic change. If this model reflects susceptibility determinants in human cancer, it will provide important insights and potential assays for studies in human populations. Furthermore, understanding determinants of susceptibility is likely to yield information concerning the molecular mechanisms of carcinogenesis.

Proposed Course: *In vivo* studies are currently underway to determine whether the cancer incidence in internal organs of SENCAR mice given carcinogens systemically is also higher than that of other strains. Preliminary studies indicate that susceptibility is confined to skin. Additional *in vivo* studies have been instituted to compare skin carcinogenesis induced by multiple application of initiator with carcinogenesis induced by a single initiating dose followed by multiple promoting doses in an attempt to determine whether the increased sensitivity of SENCAR mouse skin to carcinogenesis is at the initiation or the promotion stage. SENCAR epidermal cells, constitutively resistant to Ca^{++}-induced terminal differentiation, will be isolated for further study including their response to tumor promoters *in vitro*.

The use of the host cell reactivation system with herpes simplex virus to investigate DNA repair in epidermal cells of susceptible and resistant mice will be extended to test the ability of epidermal cells to reactivate carcinogen-treated herpes simplex virus. To examine the possibility that a deficiency in DNA repair may occur in only a subpopulation of epidermal cells, host cell reactivation experiments using constitutively initiated cell lines will be conducted. A similar approach with selected cell populations will be used with EGF binding studies. Pursuing the Langerhans cell observations, we will determine the longevity of these cells in primary epidermal cultures and observe their response to tumor promoter *in vitro*. Attempts will be made to establish cultures of pure Langerhans cells using their resistance to trypsin. This will enable seeding of cultures with known numbers of Langerhans cells. Using conditioned media and cell mixing experiments, the hypothesis will be tested that SENCAR epidermal cells produce a factor which affects the Langerhans cells function, e.g., proliferation, migration.

Further studies on the isolation and characterization of virus-specific mRNA sequences in SENCAR cells will be conducted. Specific translation products from SENCAR mRNA will be isolated and compared to BALB/c mRNA products to assess for specific gene products which may be associated with susceptibility. cDNA probes for specific sequences will be used to determine the precise nature of this RNA. By this combination of approaches, it is hoped that the elucidation of susceptibility factors and the development of susceptibility assays can be accomplished.

Publications:

Allen, P.J., Strickland, J.E., Fowler, A.K., and Waite, M.R.F.: Antigenic determinants shared by the DNA polymerase of reticulo-endotheliosis virus and mammalian type C retroviruses. *Virology* 105: 273-277, 1980.

Saviolakis, G.A., Strickland, J.E., Hellman, A., and Fowler, A.K.: Estradiol effect on type C viral gene expression in the uterus of the ovariectomized mouse. *Proc. Soc. Exp. Biol. Med.* 164: 184-191, 1980.

Strickland, J.E., Saviolakis, G.A., Weislow, O.S., Allen, P.J., Hellman, A., and Fowler, A.K.: Spontaneous adrenal tumors in the aged, ovariectomized NIH Swiss mouse without enhanced retrovirus expression. *Cancer Research* 40: 3570-3575, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05192-01 LEP

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

DNA Damage by Chemical Carcinogens and its Repair in Human Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Albert J. Fornace, Jr	Expert	LEP	NCI
OTHER:	John F. Lechner	Expert	LEP	NCI
	Roland Grafstrom	Visiting Fellow	LEP	NCI
	Curtis C. Harris	Associate Chief	LEP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.1	OTHER: .4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a new project in which we are studying DNA damage and repair caused by Chemical and physical carcinogens in cultured human cells. For example, using the alkaline elution technique, we have detected DNA-protein crosslinks in cells exposed to chromate-salts. DNA-protein crosslinks induced by trans-Pt (II) diamminedichloride appear to be repaired by the excision pathway since they are more persistent in the excision deficient cells, and because, in the presence of DNA repair polymerase inhibitor, DNA single-strand breaks only accumulate in normal cells. Comparative studies of DNA damage and repair using human bronchial epithelial and fibroblasts have been initiated. Emphasis is given to agents implicated in pulmonary carcinogenesis.

PHS-6040
(Rev. 2-81)

Objective: The purpose of our investigation is to study DNA damage induced by chemical and physical carcinogens and its repair in cultured human cells. The effect of certain chemical carcinogens on the repair of DNA damage induced by other carcinogens is also being determined.

Methods: Human fibroblasts were obtained from the American Type Culture Collection and the Human Genetic Mutant Cell Repository (Camden). Cells derived from human bronchus were maintained in culture as described in Porject Number Z01 CP 05193-01. DNA single-strand breaks and DNA crosslinks were assayed by alkaline elution. With this technique, as few as 200 DNA single-strand breaks, DNA-protein crosslinks, or DNA interstrand crosslinks per cell can be detected. DNA-protein crosslinks, produced in vitro, are measured by a filter assay which selectively binds protein and DNA crosslinked with protein but not free DNA (Braun, A., Photochem. Photo Biol. 21: 243, 1975). DNA repair replication is measured by BND chromatography (Scudiero, D., et al., Mut. Res. 29: 473-488, 1975). DNA double-strand breaks are estimated by the neutral elution method described by Bradley and Kohn (Nuc. Acid Res. 7: 793-804, 1979). Pyrimidine dimers induced by UV radiation were measured by a modification of the alkaline elution technique: Cell lysates were incubated with an extract of M. Luteus containing UV-endonuclease which has been shown to induce DNA single-strand breaks at the site of pyrimidine dimers. Alkaline elution was then carried out and the number of "UV-endo" sites estimated. With this approach, pyrimidine dimers can be measured in cells at doses of UV radiation (254 nm) of 0.02 to 1.0 j/m^2.

Major Findings: In human skin fibroblasts, mouse L1210 cells, and human bronchial epithelial cells, chromate salts induced high levels of DNA-protein crosslinks (DPC) in the same range as that induced by equal concentrations of the DNA-protein crosslinking agents such as trans-Pt(II) diamminedichloride (trans-Pt) or formaldehyde. The effect was dose dependent and was seen at low doses of potassium chromate which were not highly toxic to the cells -- colony survival greater than 20%. The DPC appeared persistent since no removal was seen with up to 18h of repair incubation. The active form of chromium appears to be the trivalent state; using either isolated nuclei or with isolated DNA and protein chromic (Cr^{+3}), compounds induced DPC but not hexavalent compounds. It is known that chromate is rapidly reduced to the chromic form in cells and that trivalent chromium forms stable coordination complexes with a slow rate of ligand exchange. A plausible interpretation of our results is that chromate salts enter the cell, are reduced to the trivalent state, and form stable coordination complexes between DNA and protein.

Further studies were performed investigating the repair of DPC in human cells. In normal human fibroblasts, DPC induced by trans-Pt (50 μM, 2h) were removed with a half-removal time of 10-12h. In excision-deficient xeroderma pigmentosum (XP) fibroblasts (Group A), DPC were more persistent with a half-removal time of greater than 24h. XP cells were significantly more sensitive to the cytotoxic effects of trans-Pt as determined by colony survival. In order to see if trans-Pt DNA damage is repaired by the excision repair pathway, cells were incubated with trans-Pt and the combination of cytosine arabinoside and hydroxyurea. This combination has been shown to inhibit the polymerase step of excision repair, but not the incision step such that after UV radiation DNA single-strand breaks accumulate in excision proficient cells. In the case of trans-Pt, DNA single strand breaks accumulated in normal cells but not XP cells -- indicating that damage induced by this agent is repaired by the excision pathway. Similar studies are currently underway with formaldehyde and chromate salts.

DNA repair studies are currently underway with cells derived from normal human bronchus. The repair of DNA single-strand breaks induced by x-rays was measured in human skin fibroblasts, bronchial epithelial cells, and fibroblasts derived from bronchus. The kinetics of repair were not significantly different in any of the three cell types and were similar to those previously published with skin fibroblasts. DNA single-strand breaks induced during the repair of UV damage was similar in these cell types. Similar levels of DNA single-strand breaks (or alkaline labile sites) were also seen in these cell types after exposure to N-methyl-N'-nitro-N-nitrosoguanidine, 7,12-dimethylbenz[a]anthracene, or (7β,8α)-dihydroxy-(9α,10α)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Repair replication after exposure to UV radiation was also comparable in fibroblasts and epithelial cells.

The effect of nickel (Ni^{+2}) on DNA repair in bronchial cells is currently under investigation. Ni^{+2} was found to have no significant effect on repair replication after exposure of either fibroblasts or epithelial cells to either UV or BPDE. Similar studies are currently underway with the various types of asbestos in bronchial cells. Excision repair is also being studied by UV-endo site removal as previously described.

Significance to Biomedical Research and the Program of the Institute: Chromate salts have been shown to be carcinogenic in laboratory animals and in human epidemiological studies. It will also transform cells *in vitro* to a malignant phenotype; another DNA-protein crosslinking agent, trans-Pt, will also induce malignant transformation. The crosslinking effect induced by chromate may play a role in its carcinogenic activity.

New approaches have been developed to study DNA repair. By use of the repair polymerase inhibitor combination (described earlier) and alkaline elution, extremely low levels of DNA single-strand breaks induced by excision repair can be detected, e.g., in normal cells excision breaks have been detected with influences of UV radiation as low as 0.04 J/m^2. The combination of *M. Luteus* endonuclease and alkaline elution allows detection of very low levels of pyrimidine dimers. Since this endonuclease preparation has apurinic endonuclease activity, it could also be applied to the detection of apurinic sites induced by chemical carcinogens.

Proposed Course: Using the bronchial epithelial cell cultures, these approaches are now being used to study DNA damage and repair with carcinogens implicated in pulmonary carcinogenesis with the clinically relevant cell type. The interactive effects between carcinogens and carcinogens in human bronchial epithelial cells will also be studied.

Publications:

Fornace, A. J. Jr., Seres, D. S., Lechner, J. F., and Harris, C. C.: DNA-protein crosslinking by chromium salts. Chemico-Biological Interactions, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05193-01 LEP

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Human Esophagus: Differentiation and Malignant Transformation at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Schlegel	Expert	LEP	NCI
Other:	John Lechner	Expert	LEP	NCI
	Herman Autrup	Staff Fellow	LEP	NCI
	Curtis Harris	Chief, HTSS and Assoc. Chief	LEP	NCI

COOPERATING UNITS (if any)
University of Maryland, Baltimore, MD; Washington Veterans Administration Hospital, Washington, D.C.; Litton Bionetics, Rockville, MD; Cancer Institute, Beijing, Peoples Republic of China

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3	PROFESSIONAL: 1	OTHER: 2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords).

The serial cultivation of human esophageal epithelial cells under clonal growth conditions has been achieved both under feeder and feeder-free conditions. Due to recent improvements in cultivation methods, the cells can be grown in the complete absence of serum. Experiments utilizing these cells in carcinogenesis studies are in progress. Cells exposed to Ni_2SO_4 gave rise to colonies with sustained growth properties. These cells are now being assessed for other properties indicative of cell transformation.

Objectives: Initial studies will be directed at developing culture conditions for the serial propagation of human esophageal cells, both under feeder and feeder-free conditions. The program of differentiation of these cultured cells will be analyzed and compared to their *in vivo* counterparts. Having established that the pattern of growth and differentiation of these cultured esophageal cells resembles that of the esophagus, these cells will be used for a variety of *in vitro* carcinogenesis studies. The ultimate goal of these studies will be the transformation of normal human esophageal epithelial cells to tumor cells using a variety of chemical carcinogens. To better understand the biology of tumor cells, we will attempt to establish various human esophageal tumors in culture. The growth properties and differentiated properties of these cells will be compared to both the normal esophageal cell and those phenotypically altered cells derived from carcinogenesis experiments.

Methods: Human esophageal tissue is obtained from "immediate" autopsy, autopsy material that is less than 3 hrs post-mortem, or occasionally from surgical specimens. The human esophageal epithelium and some adherent connective tissue is carefully dissected away from the rest of the stroma and muscular layer, minced, and trypsinized to obtain a single cell suspension. Initially, the cells have been grown by cocultivation with a lethally irradiated layer of 3T3 feeder cells in medium containing 10% fetal calf serum plus various hormonal supplements. Presently, we are developing a hormone-supplemented serum-free medium for these cells which allows for the normal growth and differentiation of these cells (similar to the cells of the natural epithelia) while under feeder-free conditions. The morphological and biochemical characterization of the cells is being assessed by a variety of techniques: light and electron microscopy, histology, histochemical staining, immunofluorescent staining, immunoprecipitation, polyacrylamide gel electrophoresis, and amino acid analysis. Subsequently, these cells will be exposed to a variety of physical and chemical carcinogens. The terminal differentiation of the normal cells will be triggered by means of ionophore or methocel-stabilized suspension culture as a selection procedure to enrich for the transformed phenotype. *In vitro* transformation will be ascertained by changes in nutrient requirements and growth properties, alterations in specific differentiation products, growth in soft agar, chromosomal changes, and tumorigenesis in athymic nude mice. The ability of normal cells and phenotypically altered cells to metabolize carcinogens, the formation of carcinogen-DNA adducts, and their purification by HPLC-chromatographic methods will be examined. Human esophageal tumors will be trypsinized to obtain a single cell suspension and plated onto dishes with or without a 3T3 feeder layer. A variety of media will be employed to try to promote the growth of these tumor cells.

Major Findings: This project was initiated for the first time within the last year. Preliminary experiments in a number of areas, however, already indicate that the human esophageal cell culture system looks like a promising model system for enhancing our understanding of carcinogenesis at the cellular level.

During the past year, we have established conditions for the serial cultivation of human esophageal epithelial cells. Initially, the cells were grown in Medium 199 supplemented with hormones and 10% fetal calf serum. In this medium, the presence of an irradiated 3T3 feeder layer was required for growth at clonal densities. The esophageal cells grew into colonies which were stratified, similar to the natural epithelia. The colonies consisted of a basal layer of mitotically active cells giving rise to the more differentiated cells of the upper cell layers. Electron microscopic examination of the cells revealed that the cells contained numerous desmosomes and tonofilaments characteristic of epithelial cells. Keratin proteins, which make up the tonofilaments, are the major differentiated product of this cell. Presently, we are extracting keratin proteins from these cells and analyzing them by polyacrylamide gel electrophoresis. To establish that the differentiated behavior of these cultured esophageal cells resembles that of the esophageal epithelium, the keratin proteins of these cells will be compared with those extracted from natural epithelia.

Experiments have also been undertaken to develop conditions which would allow for the clonal growth of esophageal cells under low serum and feeder-free conditions. After trying a variety of culture conditions, recent improvements in cultivation methods have now made possible the clonal growth of human esophageal cells under serum-free, feederless growth conditions. In addition, preliminary experiments indicate that by regulating the calcium concentration of the medium one can maintain the cultures in either a proliferating or a differentiating state. In low calcium concentration (350 M), the cells grow independently from one another as a monolayer of cells. When the calcium concentration of the medium is raised, the esophageal cells now form islands of cells capable of squamous differentiation. We are presently examining the differentiated properties of these cells under these different growth conditions using light and electron microscopy, and measuring keratin content and diversity by staining the cells with Rhodanile blue and polyacrylamide gel electrophoresis of the keratin profile.

Studies involving *in vitro* transformation of cultured human esophageal cells by a variety of *chemical* carcinogens have been initiated. Preliminary experiments indicate that the esophageal cells very actively metabolize a variety of chemical carcinogens. Presently, the carcinogen-DNA adducts which are formed are being analyzed by HPLC chromatography. Currently, esophageal cells are being exposed to a variety of chemical carcinogens. Initial attempts at *in vitro* transformation using Ni_2SO_4 look promising. Exposure of the cells *to* *10* g/ml Ni_2SO_4 (a concentration which inhibits cell growth greater than 50%) resulted in the eventual development of some epithelial colonies with altered phenotypes. Presently, the cells exposed to Ni_2SO_4 have exhibited sustained growth properties over that of control cells. These phenotypically altered cells will be characterized further in terms of altered growth properties, feeder independence, growth in soft agar, karyotype, keratin profiles, and tumorigenicity in athymic nude mice. In separate experiments, esophageal cells are also being exposed to 7,12-dimethylbenz[a]anthracene (DMBA). We also plan to expose the cells to other carcinogens and cocarcinogens. In the future, we aim to develop methods directed at selecting out phenotypically altered cells from the population of normal cells. Such methods include the triggering of terminal differentiation of the normal cells using either ionophore or methocel stabilized suspension culture.

In order to better understand the endpoint of transformation, the tumor cell, we are attempting to establish various epithelial tumors in culture. These cells will prove especially useful in helping to establish good markers of the malignant state in these epithelial cells.

Significance to Biomedical Research and the Program of the Institute: Most human cancers are epithelial in origin. A better understanding of the complex process of neoplasia will require the use of the more simplified system of cell culture. Recent advances in the ability to grow human epithelial cells in culture has now made it possible to analyze events involved in malignant transformation of epithelial cells at the cellular level.

Proposed Course: The direction of future studies will be primarily as follows: (1) optimization of conditions for growth of human esophageal cells in serum-free, feederless conditions; (2) continuation of studies aimed at understanding the control of differentiation and the sequence of events involved in malignant transformation of epithelial cells.

Publications:

None. Project recently initiated.

CONTRACT INDEX

LABORATORY OF EXPERIMENTAL PATHOLOGY
DIVISION OF CANCER CAUSE AND PREVENTION

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CONTRACT NARRATIVES
LABORATORY OF EXPERIMENTAL PATHOLOGY
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1981

EXPERIMENTAL PATHOLOGY LABORATORIES, INC. (N01-CP-95645/3 and 4)

Title: Resource for Microscopic and Autoradiographic Technology

Contractor's Project Director: Dr. Beverly Y. Cockrell

Project Director (NCI): Dr. Glennwood Trivers

Objectives: Routine preparation of biological material for light and electron microscopy.

Methods Employed: Standard electron microscopic techniques have been employed. Various embedding media have been utilized in the processing of many different sample types, including: tissue culture monolayers in plastic dishes, cell pellets, large tissue pieces, whole fetal kidneys, and tumors.

Major Findings: All data from this contract are in the form of either one micron section on glass slides or electron micrographs which are returned to the NCI originators for interpretation. In those cases where a short report of electron microscopic findings is returned to the NCI, that report is a microscopist's impression of each sample and any project findings are made at NCI.

Significance to Biomedical Research and the Program of the Institute: Electron microscopy and related techniques are valuable tools in the study of carcinogenesis and in the field of experimental pathology. Service efforts in these areas continue to haster the advancement and enhance studies within the Institute.

Proposed Course: This basic ordering agreement is to be terminated.

Date Contract Initiated: January 1, 1977.

Current Annual Level: Minimum amount - $25,589; Maximum amount - $50,645.

GEORGETOWN UNIVERSITY (N01-CP-05707)

Title: Human Tissue Collection Resource

Contractor's Project Director: Dr. Henry Yeager, Jr.

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: The main purpose of this contract is to provide the NCI with a source of human lung tissue taken at surgery, and human alveolar macrophages from fiberoptic bronchoscopy, and bronchial lavage of normal subjects, both smokers and nonsmokers.

Methods Employed: Portions of human bronchus and/or peripheral lung are taken in as sterile a fashion as possible when available from Surgical Pathology, Georgetown University Hospital. Lung/bronchus specimens are placed directly into sterile Lebowitz medium and transported on ice to NIH. Lung macrophages are harvested from normal volunteers by fiberoptic bronchoscopy and bronchial lavage. Volunteers are screened for normal lung function using standard pulmonary function tests and lung X-ray (PA and lateral). Macrophage viability is assessed using trypan blue exclusion. Cells are suspended in RPMI-1640 supplemented with fetal calf serum, penicillin and streptomycin, placed on ice, and sent directly to NIH.

Major Findings: A total of ten surgical lung samples have been provided to the NCI in a ten-and-one-half month period, from the time that the contract was actually activated at Georgetown. (Six weeks were necessary to get the technician position approved and to hire an assistant at Georgetown.) Sixteen samples of alveolar macrophages from fiberoptic bronchoscopy have also been provided to the NCI. The macrophages were from smoking and nonsmoking normal volunteers. These materials were provided for experiments to be carried out at the NCI.

Significance to Biomedical Research and the Program of the Institute: The contractor has been able to make human lung material available to the NCI on a regular basis to conduct studies of carcinogenesis.

Proposed Course: To continue to provide human lung tissue and alveolar macrophages to the NCI for the remainder of the contract period.

Date Contract Initiated: May 1980.

Current Annual Level: $52,000.

LITTON BIONETICS, INC. (NO1-CP-43274)

Title: Xenotransplantation Resource for Studies of Carcinogenesis in Human Tissues

Contractor's Project Director: Dr. Marion G. Valerio

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: To use an immunodeficient animal model, the athymic nude mouse, for long-term survival of human tissue xenografts; to provide a continuing resource of athymic nude mice for these long-term studies; to use human tissues to study the development of preneoplastic and possibly neoplastic lesions induced by carcinogens and to study the ability of selected agents to modify the effects of carcinogens on human tissue.

Methods Employed: The human epithelial tissues are exposed to carcinogens either prior to or after transplantation and maintained *in vivo* in the athymic nude mouse as xenografts. Human cells are injected in various sites. A self-sustained breeding colony of athymic nude mice is maintained in a modified barrier facility.

Major Findings: Human bronchus, pancreatic duct, colon, breast, prostate and esophagus can be maintained for long periods of time as xenografts in the nude mouse as evidenced by a viable-appearing epithelium with normal histology and the incorporation of tritiated thymidine into epithelial cells of the grafts. Squamous metaplasia has occurred in graphs treated in vivo to several carcinogens, but these have not progressed under current treatment regimens.

Significance to Biomedical Research and the Program of the Institute: The establishment of an animal host for human tissue xenografts which have been treated with carcinogens and/or anticarcinogens in vitro and/or in vivo will provide a model more predictive of the effects of carcinogens and anti-carcinogens on human tissues than extrapolation from an animal tumor or tissue culture model system. This sytem provides an opportunity to study chemical carcinogenesis in the target tissues for human cancer.

Proposed Course: This contract will terminate on June 30, 1981 and the effort will be recompeted.

Date Contract Initiated: February 1, 1974.

Current Annual Level: $240,000

MARYLAND, UNIVERSITY OF (N01-CP-15736; successor to N01-CP-95640)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Contractor's Project Director: Dr. Benjamin F. Trump

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: The objectives of this program are to provide a resource to the National Cancer Institute for the obtainment, transport, and characterization of normal, preneoplastic tissue from the human bronchus, pancreatic duct, colon, and liver.

Methods Employed: Neoplastic and non-neoplastic tissues from human bronchial epithelium, pancreas, colon and liver are collected at time of surgery and autopsy, including immediate autopsy. Samples are processed for morphological study including light and electron microscopic study, histochemistry and immunocytochemistry. Samples are also collected for organ (explant) and cell culture, metabolic studies and measurement of ion ratios.

Major Findings:

Bronchus: 1) Morphological and histochemical studies of human primary lung carcinomas are continuing in collaboration with Dr. A. Apostolides, Department of Epidemiology and Preventive Medicine at the University of Maryland School of Medicine. Epidemiologic data are being analyzed to study the relationships between lung tumor type, selected risk factors and the amount of benzo[a]pyrene (BaP) bound to DNA by the same patient's non-cancerous bronchial epithelium. To date the data suggest that higher binding levels of BaP are found in bronchi from patients with tumors showing epidermoid differentiation than from patients with tumors showing well-differentiated adenocarcinoma differentiation with no epidermoid component. In additional studies, the binding of BaP to monocyte DNA is being measured and correlated with the amount of BaP bound to DNA of the bronchial epithelium of the same patient and with other epidemiological data.

2) Immunohistochemical characterization of the human tissues has continued using the peroxidase-antiperoxidase method to demonstrate the presence or absence of various antigens. The tissues being examined include lung tumors, normal fetal lung and normal, abnormal and preneoplastic adult bronchus. Markers include human chorionic gonadotropin (HCG), calcitonin, adrenocorticotropic hormone (ACTH), serotonin, alpha-fetoprotein (AFP), keratin, somatostatin, and neuron specific enolase (NSE). HCG appears to be an oncofetal antigen, being present in the fetus, absent in the adult without cancer and present in some early neoplastic lesions as well as in 80% of lung tumors studied. The contractor has confirmed the observation of others that calcitonin is present in isolated fetal epithelial cells and has found that it is present in some 20% of tumors as well. ACTH has been demonstrated in 40% of the tumors studied. Serotonin and NSE have been found only in tumors containing endocrine granules. Keratin has been observed in 80% of lung tumors, including some without noticeable keratin filaments, indicating that keratin may exsist in an unpolymerized state in tumors. Keratin has also been demonstrated in fetal lung and metaplastic adult bronchus. Somatostatin has been found in some 40% of lung tumors, but is often present only focally. AFP has been found in only one lung tumor.

Characterization of the microtubule system of the cytoskeleton in normal and neoplastic human cells is continuing. Epithelial cell outgrowths are obtained on cover slips from surgical tissue explants and are used for immunofluorescent and immunohistochemical studies. Immunofluorescent observations of microtubules in malignant cells reveal consistent differences in pattern when compared with normal cells. The normal cells are generally of uniform size and display mostly straight microtubules originating from assembly sites near the nucleus, whereas tumor cells are of mesh-like arrangement. Preliminary work has also been done using immunofluorescence and antibodies for both DNase I and calmodulin on these epithelial cell outgrowths.

3) Tracheo-bronchial epithelium obtained from routine autopsies may possibly be useful as a material to generate primary epithelial cell cultures. Cell culture can be used in several areas within the overall program. For example, cell cultures are used to compare the microtubular pattern, as demonstrated by immunofluorescent staining, between normal and malignant cells.

Pancreas: Pancreatic tissues obtained at the immediate autopsy are being examined by morphological techniques including light and electron microscopy, histochemistry, immunohistochemistry and freeze fracture. Pancreatic ducts are being studied by organ explant and cell culture techniques developed in our laboratories. We have also obtained tissues at routine autopsies and from surgical procedures. These cases are also being examined further to elucidate cellular alterations in human pancreatic cancer.

Colon: 1) Morphological (LM, TEM, SEM) and histochemical examinations of normal, premalignant and malignant human epithelium are continuing. A comprehensive description of the morphological features of the various segments of the normal human colon has been prepared. The light microscopic, histochemical and electron microscopic features of the four segments (ascending, transverse, descending, rectum) are different. Indeed, some of the previously reported morphological markers of premalignant changes may actually represent normal features of the different segments.

2) Human ascending, transverse and rectal colonic epithelium from immediate autopsies are being maintained routinely in explant culture and provided to the NCI for xenotransplantation.

Liver: Various methods for the primary culture of human hepatocytes and rat hepatocytes are being compared. The contractor has obtained all the reagents needed to make the liver biomatrix, which has recently been shown to enhance cell survival in culture. To date, one of three isolations performed on immediate autopsy cases has been successful in obtaining viable human hepatocytes. Cultures were maintained for eight days. On non-successful cases, subsequent EM examinations of zero time specimens showed irreversible cell injury, indicating cells were dead prior to isolation.

Significance to Biomedical Research and the Program of the Institute: Of significance to the NCI program and biomedical research is the pressing need to develop systems in which data can be obtained on the response of important epithelial tissues to toxic and carcinogenic stimuli. This is essential in order to provide methods of interspecies comparisons and extrapolation of animal data to man. It is essential to continue the development and characterization of systems for long-term maintenance and study of interactions of various environmental stimuli with normal human tissues obtained from individuals with non-neoplastic disease. Characterization of such tissues from a variety of standpoints is necessary to assess viability, determine reversibility, assess development of markers for normal, preneoplastic, and neoplastic tissues and to examine a variety of influences on carcinogen metabolism. Such developments will not only improve the study of human tissues but will assist in future development of improved animal models.

Proposed Course: During the coming year, emphasis will continue to be placed on providing this unique resource to the NCI.

Date Contract Initiated: February 1, 1981.

Current Annual Level: $408,001.

MARYLAND, University of (NO1-CP-75909)

Title: Studies of Carcinogenesis in Human Tissue

Contractor's Project Director: Dr. Benjamin F. Trump

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: To provide specimens of human esophagus to the Laboratory of Experimental Pathology for carcinogenesis studies.

Major Findings: Methods to obtain, store, transport and culture human esophagus have been utilized. Forty specimens have been collected and characterized by the contractor. An epidemiological profile has been obtained from the donors. The contractor has examined the baseline (zero-time) morphological characteristics of normal, premalignant and malignant esophageal epithelium. By light microscopy, the tumors were diagnosed as squamous cell carcinomas with varying degrees of differentiation. Adeno characteristics were revealed upon electron microscopic examination. An interesting finding was the presence of numerous intracytoplasmic desmosomes in several tumors.

The contractor has examined the morphological characteristics of normal human esophageal epithelium prior to and for extended periods of time in organ culture (7-181 days). In this culture system, the squamous epithelial characteristics were retained as demonstrated by light and electron microscopy. In the Laboratory of Experimental Pathology human esophageal epithelium in vitro is capable of metabolizing chemical carcinogens such as benzo[a]pyrene and N-nitrosodimethylamine. Control human esophageal explants can be successfully xenografted into the athymic nude mice.

Significance to Biomedical Research and the Program of the Institute: The goal of this project is to study carcinogenesis in human esophageal epithelium. Such studies are important not only for understanding the detailed pathogenesis of the human lesion and for development of methods for the testing of carcinogens in vivo and in vitro, but will also serve as models for the development of similar methodologies in other tissues. The methods and concepts developed in this program can then be used by other groups to foster the further development of carcinogenesis research in human tissues.

Proposed Course: Continuation of current tasks.

Date Contract Initiated: September 30, 1977.

Current Annual Level: $50,000.

MELOY LABORATORIES, INC. (N01-CP-15766; successor to N01-CP-95622)

Title: Resources for Transplacental Carcinogenesis in Primates

Contractor's Project Director: Dr. D. Lewis Sly

Project Officer (NCI): Jerry M. Rice, Ph.D.

Objectives: This project is designed to provide a non-human primate model to demonstrate and study transplacental chemical carcinogenesis.

Methods Employed: Injections of chemical carcinogens. Surgery. Clinical and histopathological assays.

Major Findings: Pregnant monkeys exposed to ethylnitrosourea have shown that the period of maximal fetal susceptibility to this chemical appears to be early in gestation. Tumors have been seen most frequently in babies first exposed to ENU at approximately 30 days of gestation. These tumors have had short latencies with most developing before 6 months of age. Tumors from animals exposed later in gestation have occurred later in life with lower frequency. The tumor types seen in these transplacentally exposed primates differ markedly from the tumors seen in rodent studies. Sarcomas of various organ systems have been the primary finding, rather than carcinomas as seen in mice or neurogenic tumors as in rats. In addition embryonal kidney tumors and hepatocellular carcinomas which produce α-fetoprotein have been induced transplacentally. Tumors have also been seen in juvenile and less frequently in adult animals inoculated with ENU. Pregnant females, however, have shown enhanced susceptibility to ENU due to the risk of placental neoplasms.

Significance to Biomedical Research and the Program of the Institute: With recognition of the association between exposure to diethylstibestrol in utero and development of vaginal adenocarcinoma during the second decade of life, the possible significance to human health of transplacental chemical carcinogens has become increasingly a matter of concern. Experimental studies in transplacental chemical carcinogenesis have been limited to rodent species, which appear to differ significantly from man in ways likely to be of importance for understanding the different consequences of exposure to chemical carcinogens during fetal versus adult life. Among these differences are the more rapid rates of fetal and neonatal development and maturation in rodents. The short latencies for tumor appearance and the type of tumors observed to date in the Patas monkey provide experimental support for the position that at least some tumors of infancy and childhood, including certain congenital tumors, may result from prenatal exposure to carcinogens.

Proposed Course: Animals previously exposed transplacentally to carcinogens will be monitored for tumor development and tumor type as they progress in age. In addition, a variety of studies is being initiated to explore the co-carcinogenic effects of several tumor promoting agents. Both phenobarbital and asbestos have been shown to promote tumorigenesis in rodent studies. The question of whether these types of agents, which are present in the human environment, also intensify or accelerate tumors in primates and in man is extremely important. These studies will determine if limited prenatal exposure to carcinogens previously shown to produce sarcomas in monkeys will, with subsequent exposure to a tumor promoting agent, give rise to carcinomas of lining epithelium. Epithelial carcinomas are the major form of cancer in man. The role played by these tumor promoting agents in man can be investigated by these studies in another primate species.

Date Contract Initiated: November 23, 1978.

Current Annual Level: $234,589.

MICROBIOLOGICAL ASSOCIATES, Inc. (N01-CP-05637)

Title: Biochemistry and Cell Culture Resource

Contractor's Project Director: Dr. Rodger D. Curren

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: The purpose of this contract is to provide the Laboratory of Experimental Pathology at the NCI with resources (e.g., retinylphosphate, feeder cells, conditioned media, and human bronchus cells) as necessary to conduct investigations of chemical carcinogenesis in general, and the mechanisms of human carcinogenesis in particular.

Methods Employed: This is a multifaceted contract serving four different research areas of the Laboratory. The methods used are provided by the individual program personnel and involve synthesizing biologically active molecules, culturing cell lines from rodent and human sources, analyzing the *in vitro* effects of known and suspected carcinogens, and determining the biochemical nature of carcinogen metabolites.

Major Findings:

A. Synthesis of Retinylphosphate

During the period from July 1, 1980 to June 1, 1981, 401 mg of retinylphosphate have been synthesized and delivered to the NCI. When this project was initiated,the synthesis and separation methodology gave a product yield of approximately 8%. During the course of the year several steps in the procedure were optimized, resulting in yields which commonly reach 20-25%. Three additional compounds, mono-ene perhydroretinol, phorbol, and the methyl ester of dihydroxyretinoic acid, were phosphorylated by the same methods.

B. BALB/c 3T3 Transformation

During the last year the contractor has 1) characterized, expanded and cryopreserved (approximately 250 ampules) four lines of BALB/c 3T3 cells; 2) tested 16 lots of serum for the ability to support a high plating efficiency, an acceptable cell saturation density, and a significant level of chemically induced phenotypic transformation; and 3) initiated 33 transformation assays using protocols provided by NCI staff. Transformation assays with model chemicals have demonstrated the following:

a) A clone of 3T3 cells which exhibits little spontaneous transformation
b) Dose-response with chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), benzo(a)pyrene (BaP), aflatoxin B_1, and benzidine
c) Sensitivity to BaP as low as 0.002 g/ml
d) An optimum exposure time to BaP of 24-72 hours
e) An optimum exposure time to MNNG of 24 hours
f) An optimum exposure time to aflatoxin B_1 of 24 hours
g) An optimum exposure time to benzidine of 72 hours

C. Mouse Epidermal Cell Culture

Three phases of this work are continuing. The first phase consists of preparing conditioned media. This media is necessary for the growth of cloned mouse epidermal cells. To date the contractor has produced 27 liters of fibroblast-conditioned media which have passed all sterility and growth promotion tests. Additional media have been produced which were rejected for one reason or another.

A second phase is the growth and storage of BK-1 "superclone" cells. So far approximately 60 ampules at 2 x 10^6 cells/ampule have been cryopreserved. We have tested the ability of these cells to metabolize BaP to water soluble forms. To date, two tests have shown these cells (at least at passage 12) to have much less metabolizing activity than Swiss 3T3 cells or human fibroblasts.

A third phase of work involves developing quantitative assays using BK-1 cells for the loss of calcium-induced terminal differentiation. Three dose-response assays using MNNG have been initiated to this time.

D. Human Bronchus Culture

The contractor has been growing and cryopreserving both human bronchial epithelial cells and human bronchial fibroblasts. In order to expand the epithelial population to a size at which it can be frozen, large amounts of Swiss mouse 3T3 cells are needed for feeder layers. To date, the contractor has produced, irradiated, and delivered 1.1 x 10^{10} cells (twice weekly) to the NCI, plus approximately 10-20% of that amount for use by the contractor.

The contractor has cryopreserved 140 x 10^6 bronchial epithelial cells from 7 different donors. In addition, bronchus explants from four of these donors have been used to generate fibroblasts (300 x 10^6) which have been frozen down at various passage levels.

E. Cell-mediated Mutagenesis

A human lymphoblastoid cell line is being used as a target cell in a human epithelial cell-mediated mutagenesis assay. Using selective conditions necessary for detecting mutations resulting in a OUA^r or $6\text{-}TG^r$ phenotype, several dose-response assays have been conducted with a direct acting mutagen, MNNG, but results with a promutagen, BaP, have been less successful. Although several positive results both with cytotoxicity and induced mutations have been found when human bronchus cells are used as a metabolizing feeder layer, the overall results are still inconsistent and the experimental conditions are being modified to improve the reliability of the assay.

F. Biochemistry of Carcinogen Metabolism

This section of the contract has been involved mainly with HPLC analysis of BaP metabolites, separation of BaP conjugates on alumina columns, and extracting DNA from tissue samples and measuring the H^3-BaP binding levels. So far this year the contractor has analyzed 125 media samples by HPLC, has processed 230 media samples on alumina columns, and has extracted the DNA from 28 tissue samples and measured the BaP binding to the DNA.

Significance to Biomedical Research and the Program of the Institute: These support services enable the Laboratory of Experimental Pathology to better carry out investigations that deal primarily with mechanisms of in vitro chemical carcinogenesis in both animal and human tissue. An understanding of the similarities and differences between animal and human tissue in vitro may lead to answers of the more important questions concerning the similarities and differences in the response of animals and humans in vivo to chemical carcinogens.

Propose Course: Continue the above tasks.

Date Contract Initiated: July 1, 1980.

Current Annual Level: $398,912.

MICROBIOLOGICAL ASSOCIATES, Inc. (NO1-CP-15744; successor to NO1-CP-02199)

Title: Laboratory Rodent and Rabbit Facility as a Resource to the Laboratory of Experimental Pathology

Contractor's Project Director: Martin L. Wenk, Ph.D.

Project Officer (NCI): Jerry M. Rice, Ph.D.

Objectives: The purpose of this contract is to provide support services for the Carcinogenesis Intramural Program of NCI in carrying out long-term treatment, holding, and observation of animals in carcinogenesis investigations emphasizing life-time tumor induction in rodents, and related activities.

Methods Employed: American Association for the Accreditation of Laboratory Animal Care accredited animal housing facilities, general supportive laboratory facilities, qualified personnel, materials and equipment not otherwise provided by the government are employed to carry out protocols developed in collaboration with intramural NCI investigators and approved by the NCI Project Officer. Such protocols are carried out by the use of contract resources for the following functions: 1) the preparation, handling, and administration of chemical solutions according to NCI guidelines for the safety of personnel carrying out research involving chemical carcinogens; 2) the holding, treatment and data collection (including gross pathology data) for mice, athymic nude mice, hamsters, rats, guinea pigs, and rabbits; 3) the administration of chemical carcinogens to animals by skin painting, gavage, parenteral injection, intratracheal administration or other protocol dictated routes; 4) the feeding of commercially pelleted or meal form diets as protocolled or experimental diets as required; 5) the storage of labile animal diets, reagents, tissues, or other materials under conditions of temperature regulation (4°C, -20°C, -70°C, or liquid nitrogen); 6) the qualitative or quantitative analyses of carcinogen preparations, tissues of carcinogen-treated animals and other biochemical investigations as required; and 7) the preservation and supply according to protocol of tissue samples to be evaluated by light microscopy, electron microscopy, radioautography, biochemistry, or histochemistry at NCI.

Major Findings: Since the inception of this contract, December 30, 1980, support has been made available to 26 NCI investigators in conducting 25 individual experimental protocols. Contract efforts were concentrated in the following areas: 1) the development of in vivo animal models for neoplasms relevant to the human population; 2) the utilization or devlopment of model systems as bioassays for the determination of chemical carcinogenicity; 3) the utilization of model systems to determine the pathogenesis of chemically induced neoplasms; 4) the utilization of model systems to evaluate the modifying influence of various factors upon chemical carcinogenesis (factors include age, sex, or genetically determined susceptibility, target organ cell cycle kinetics, antineoplastic compounds, natural or synthetic dietary additives, route of exposure, and influence of exposure level; and 5) the application of contract resources to support intramural NCI programs in experimental pathology. Support in this last category includes the evaluation of new experimental procedures, the supply of immunological reagents, the breeding or holding of animals not routinely available through other sources, the availability of a nude mouse colony for various research projects, and the availability of general laboratory facilities for the support of protocolled experimentation.

Significance to Biomedical Research and the Program of the Institute: This contract offers facilities, expertise, and manpower to support the ongoing and developing scientific programs of the Carcinogenesis Intramural Program. The ability to conduct rapid tests of new approaches in experimental pathology, to conduct both short- or long-term in vivo experimentation involving large numbers of animals, and to rapidly initiate studies that are relevant to the overall objectives of the Carcinogenesis Program are critical to the Institute's scientific program.

Proposed Course: To continue to offer a diversified facility for the support of ongoing scientific programs within the Carcinogenesis Program and to develop resources and capabilities as their need is deemed necessary.

Date Contract Initiated: December 30, 1980.

Current Annual Level: $611,171.

NATIONAL NAVAL MEDICAL CENTER (Y01-CP-80204)

Title: Procurement of Human Tissues

Contractor's Project Director: Dr. R. Zajtchuk

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: To provide preoperative histories and nontumorous bronchial epithelium (obtained at the time of surgery for lung cancer or for benign lesions) to the NIH for the study of carcinogen activation and deactivation, and the capability to metabolize carcinogens to mutagens.

Methods Employed: Using the OR schedules for the following day, a medical biology technician contacts the attending physician(s) for permission to seek informed consent and medical history information from subject patients. On the day of surgery, the technician notifies the NCI of a pending specimen, arranges with the pathologist for assistance in collecting the tissue and provides a sterile container and cold L-15 medium. With the surgeon's permission and assistance, the technician obtains human tissue, transfers the specimens to the pathologist for sterile dissection of the nontumorous tissue, then immediately transfers the material to NCI researchers for final dissection and subsequent culturing.

Major Findings: Since the initiation of this agreement on October 2, 1978, the National Naval Medical Center (NNMC) has delivered a total of 116 surgical specimens of human lung (20) and colon (96) to the NCI. Appropriate clinical history has been obtained on all patients and the specimens have been properly processed through the Pathology Department of the NNMC. In addition to these 116 patients, another 23 have been obtained, but have either not had cancer or were not resectable; therefore, no specimens were available.

Significance to Biomedical Research and the Program of the Institute: Lung and colon cancer are among the most frequent types affecting the human population. They are also among the forms of cancer most likely to be influenced by environmental factors such as chemicals; therefore, it is important to determine whether or not persons with diagnosed disease or who are suspected of preneoplastic changes suffer a predisposition or increased susceptibility to the transformational effects of chemical carcinogens. Research of this nature has potential for both cancer detection and cancer therapy.

Proposed Course: It is anticipated that the contractor will continue to perform at his established or a somewhat higher level of volume.

Date Interagency Agreement Initiated: October 2, 1978.

Current Annual Level: $15,150.

VETERANS ADMINISTRATION HOSPITAL (Y01-CP-60204)

Title: Studies on Normal, Premalignant and Malignant Respiratory Epithelium of Humans

Contractor's Project Director: Paul W. Schafer, M.D.

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: The general objectives of this interagency agreement are (a) to affect a better morphologic and biochemical characterization of normal, premalignant and malignant respiratory epithelium; (b) to obtain and establish organ cultures of essentially normal human lung tissues so that they may be studied in regard to their response to carcinogens both in culture and by xenotransplantation into immune deficient experimental animals; (c) to study the effects of chemotherapeutic or antineoplastic agents on evolving pre-malignant or *in situ* malignant changes induced in experimental animals by cytochemical, light and electron microscopic techniques; (d) to structure a lung cancer classification based on coordination of input from all collaborations; and (e) to extend the above undertakings to esophagus and colon.

Methods Employed: An abstract of the clinical record is prepared for each patient whose tissues are entered into the study. The patient's specific problem requiring operative intervention is therein identified. Results of specific preoperative diagnostic procedures are included together with such assessments of pulmonary functions as were undertaken to evaluate the patient's ability to withstand surgery. Pulmonary disease other than that for which the surgery is performed is also identified. Sputum, brush and lavage specimens and biopsies are characterized morphologically.

Gross pathology encountered at surgery and pertinent aspects of the operative procedures are entered in the input protocol. The operative procedures employed range from local excisions, through segmental or complete lobectomy and bi-lobectomy, to total pneumonectomy. Only those tissues are resected that are required for diagnostic and therapeutic purposes. With a tissue proven preoperative diagnosis, a definitive resection without further biopsy is performed when technically possible. In the absence of a positive preoperative diagnosis, the diagnostic procedure of choice has been a total local excision, and only when this is not technically possible is incisional biopsy used. No tissues are obtained solely for the purposes of this investigation.

All resected specimens are asceptically dissected promptly upon removal from the patient. Parts of the specimen critical to the requirement of the Veterans Adminstration Hospital laboratory service, particularly those tissues at the line of resection, are left untampered. As much of the bronchial tree as otherwise could be obtained are dissected free. Adequate samples are taken of all disease processes encountered. Representative samples of the foregoing are variously prepared for light and electron microscopy. The remainder are placed in L-15 medium and stored at 4°C for transport to the NCI for culturing and subsequent xenotransplantation studies.

Fixed tissue, stained and unstained sections, as well as multiple touch preparations are also made available for pathological examination.

Major Findings: The contractor continues to obtain tissue samples for study. Lobar, segmental and sub-segmental bronchi have been recovered from 88 patients who underwent the following operations:

Right pneumonectomy	10
Left pneumonectomy	9
Right upper lobectomy	30
Right middle lobectomy	3
Right lower lobectomy	10
Left upper lobectomy	7
Lingulectomy	2
Left lower lobectomy	10
Bilobectomy	3
Local excision	4
Total	88

By light microscopy it has been ascertained that five of the 88 patients did not harbor a malignancy but rather had respectively, aspergillosis, crypotococcosis sequestration, bronchiectasis and granuloma. The 83 pulmonary malignancies have been identified as follows:

Squamous cell carcinoma	53
Adenocarcinoma	13
Undifferentiated carcinoma	10

Broncho-alveolar carcinoma	3
Carcinoid	3
Carcinoma *in situ*	1
Total	83

Significance to Biomedical Research and the Program of the Institute: Model systems incorporating human tissues in culture or in experimental hosts are promising targets for experimental carcinogenesis by suspect classes of environmental agents. Ultimately, they may come to represent the basis for development of new prophylactic and definitive therapies.

Proposed Course: Based on mutual experiences to date, the contractor's role in the lung carcinogenesis collaboration should continue as follows:

1. Continued obtainment of viable normal and abnormal human pulmonary tissues under those strict medical, legal and ethical structures which characterize human experimentation.

2. Visible light and electron microscopic characterization of the foregoing.

3. Enlarged collaboration in the maintenance of these tissues *in vitro*, then xenotransplantation into immunodeficient animals, and then experimental treatment in both settings.

4. These obtainments will be increased to include more esophageal, gastric and colonic mucosa when possible in the course of resections.

Date Interagency Agreement Initiated: March 1976.

Current Annual Level: $49,370.

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SUMMARY REPORT

LABORATORY OF MOLECULAR CARCINOGENESIS

October 1, 1980 to September 30, 1981

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts a research program designed to (1) clarify the molecular biology of carcinogenesis; (2) elucidate the fundamental nature of the interaction of carcinogenic agents, especially chemical, with biological systems in the induction of cancer; (3) define those environmental and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the metabolic regulatory processes which are related to carcinogenesis.

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research program is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation and identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The Laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular and organism levels and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. These processes are studied in biological preparations and cells from experimental animals and humans.

Cancer is a disease in which the genetic information and/or expression is altered. This altered phenotypic expression may be due to genetic or epigenetic events induced by either xenobiotic or endogenous factors which result in altered patterns of gene control. The central aim of the Laboratory is to understand how exogenous carcinogens and endogenous factors are processed by enzymatic mechanisms, how the carcinogen is converted to active forms and the nature of the interaction between the latter and the gene action system. Systems involved in the repair of genetic damage induced by carcinogens are studied. Understanding is sought on how the interaction with carcinogens results in the modifications which characterize malignant transformation. The aim of these studies is modification, elimination or prophylaxis of the carcinogenic response in the human population.

During the past few years there have been important new ideas about the molecular mechanisms involved in chemical carcinogenesis. In the past, most research centered around the view that the most important consequence of exposure to chemical carcinogens was base change mutation; now the idea that rather extensive rearrangement of the genome without significant base alteration can lead to malignancy has become tenable. These concepts derive principally from detailed sequence studies of transforming genes in virally induced cancer. It is clear that many of these genes are normal cellular genes which find increased expression by reason of either increased copy number, or increased transcription as a consequence of proximity to a stronger promoter. At the same time, it has been found that chemical carcinogens, in addition to their ability to modify the bases in the DNA chain, can lead to increased rates of gene amplification and translation of genes to new sites in the genome. The Laboratory has been strengthened in this area by the addition of new personnel especially well trained in the molecular biology techniques involved in the latter studies. It is my expectation that these new skills will be acquired by others and generally applied in the Laboratory. Several projects related to these ideas have been undertaken, and several more will be initiated in the coming year.

The use of monoclonal antibodies is a second major new tool which the Laboratory is applying to the study of carcinogenesis. The specificity of the information obtained with these reagents may well prove complementary to the base sequence data that is being developed. These techniques offer special promise in characterizing the genetic basis for carcinogen susceptibility. Our reports this year indicate several areas where these techniques have been applied.

The potential of the new biology truely staggers the imagination, and we hope to develop sets of research problems with senior and support personnel teams which can maximally exploit these new techniques and ideas for the understanding of the molecular basis of chemically induced cancer. The emergence of these extraordinary and powerful new techniques and new ideas presents a challenge as to the most effective organization to match personnel, equipment, and problems. We have begun explorations of possible ways in which our highly skilled and imaginative investigators can benefit each other in the most productive way. While we sense the significance of this problem, more thought must be given to it before a solution good enough for the next decade can be offered.

Cell Genetics Section - Studies (1) the molecular mechanism of initiation of malignant transformation of cells by chemical carcinogens and radiation; (2) the cancer prone genetic diseases; (3) the structure and function of cell lar genes and their products involved in the expression of the transformed state.

The aim is to understand the molecular events governing the malignant transformation of cells by chemical carcinogens and radiation. Research in the past decade has provided much fundamental information on the very early steps of carcinogenesis, such as metabolism of carcinogens and binding of carcinogens to DNA. However, the crucial processes of cell transformation after the interaction of ultimate carcinogens with cellular molecules are still quite vague. This Section attempts to identify the cellular molecules or metabolism directly involved in these crucial processes of cell transformation. Current primary areas of research in this Section are: 1) development of an optimum system for studying malignant transformation and mutation of cultured cells including human cells by chemical carcinogens; 2) investigation of the genetic and physiological factors affecting cell transformation with special attention to DNA repair function and promotor-effective function; 3) mechanism of cellular hypersensitivity of cancer prone genetic disease patients and its relation to clinical abnormalities; 4) cellular and molecular effects of psoralen plus long wavelength ultraviolet radiation (UV-A); 5) mechanism of alteration of gene expression associated with the malignant transformation and identification of macromolecules involved in the expression of the transformed state; 6) mechanism of promotion of cell transformation by promoting agents.

In order to provide an optimum system for studying the mechanism of cell transformation, attempts are being made to develop a rapid, reliable, convenient, and quantitative system for assay of malignant transformation and mutation of mammalian cells. Consequences of this research may be the discovery of the crucial factors involved in cell transformation and the development of an improved assay system for assessment of human risk against environmental carcinogens. Skin fibroblasts derived from patients with familial polyposis and Bloom's syndrome were examined for their behavior in culture and were found not to be unique compared to normal human skin fibroblasts. Studies are in progress to determine whether cells from these patients who are genetically predisposed to a higher incidence of cancer are highly sensitive to transformation as well as mutation. A protocol for a quantitative and efficient system for the assay for dominant mutations of human diploid fibroblasts was established using a diphtheria-toxin resistance marker. The factors contained in the serum which markedly affect the frequencies of chemically-induced transformation are being investigated.

Cell variants of Balb/3T3 line showing different susceptibility for transformation have been previously isolated by us and are being characterized as one of the approaches to identify the genetic factors affecting cell transformation. We found that the fixation of transformation by carcinogens occurs at a similar high efficiency in all the variant cells and that variants are different in the expression process by which promoting agents act and may be the basis for the differential susceptibility to transformation between variant cells. These results provide the first genetic evidence for the important role of a promoter-responsive step in the induction of cell transformation. The mechanism of the promotion step is being studied using these variants.

In another approach to identify the genetic factors involved in cell transformation, human cancer-prone genetic diseases are being studied with special attention to 1) correlation of cellular hypersensitivity with clinical abnormalities, 2) genetic diversity within such groups, and 3) the molecular basis for cellular hypersensitivity. An extremely comprehensive review has been compiled on the clinical features and laboratory abnormalities in xeroderma pigmentosum (XP). Establishment of a registry of XP patients in the United States and an attempt to clone the genes responsible for UV sensisitivity of XP cells are in progress. We found that ataxia telangiectasia lymphoid cells are abnormally sensitive to bleomycin measured in terms of cell killing and induced chromosome breaks. Other research in progress includes a clinical definition of familial malignant melanoma with dysplastic nevi, studies of survival of cultured cells from dysplastic nevus syndrome patients, and determination of susceptibility of cells from familial polyposis and Bloom's syndrome patients.

Photochemical carcinogenesis with relevance to clinical therapies is under investigation. Psoralen plus long wavelength ultraviolet radiation (UV-A) is used experimentally in treatment of psoriasis but has been found to be carcinogenic in mice and humans. It was found that 8-MOP plus UV-A results in decreased DNA systhesis, DNA-8MOP cross-link formation, cell killing, increased sister chromatid exchanges and decreased mixed leukocyte reactivity.

In parallel with an attempt to identify the cellular molecules responsible for the expression of the transformed phenotype, the mechanism of polypeptide changes associated with the transformation of human cells has been investigated. Some progress has been achieved in this area. When the protein molecules of the chemically-transformed human fibroblasts and normal parent cell, were compared by two-dimensional gel electrophoresis, approximately 20 polypeptides were different out of more than 1000 distinguishable polypeptides. One new polypeptide recognized in the transformed cells was identified as a product of a mutated β-actin gene by immunoprecipitation with anti-actin antibody, comparison of its tryptic peptide patterns, isolation of mRNA coding for the new protein, cloning of DNA complementary to actin mRNA from the transformed cells into pBR322, determination of size of actin mRNAs, complete amino acid sequencing of normal and altered β-actin molecules, and Southern blotting analysis of the actin gene in the transformed cells using a recombinant DNA actin cDNA. These results provide the first molecular evidence for the occurrence of a mutation in the chemically transformed cells. Some results were obtained which indicate a close correlation of the production of a mutated β-actin with the expression of the transformed phenotypes.

The mechanism of promotion of chemically induced transformation by promoting agents is being investigated. First, it was found that post-treatment with promoting agents results in the marked enhancement of carcinogen-induced cell transformation in the Balb/3T3 subclones as reported in other transformation systems. A naturally occurring promoter, dihydroteleocidin B (DHTB), was found 100-fold stronger in the ability to enhance carcinogen-induced transformation than TPA. Biochemical studies on the cellular response to promoter revealed that an extraordinary enhancement of transformation by DHTB is ascribed to its resistance to metabolic inactivation which results in the continuous exposure of the cells to an active form of the promoting agent. Many biochemical changes induced by a single application of promoting agents disappeared after a few repeated applications of promoters due to down regulation. These results suggest that biochemical changes induced by a single application of promoter may not be directly involved in the promoting process. Further studies are in progress using Balb/3T3 cell variants which differ in the promotor-effective process.

Metabolic Control Section - Studies (1) the metabolic effects on tissues and cells resulting from their exposure to chemical carcinogens and the relationship between these effects and malignant transformation; (2) the metabolic activation and detoxification of the polycyclic hydrocarbons and the relationship of this metabolism to individual susceptibility; (3) regulation, and structure of the genes for the enzymatic system primarily responsible for the metabolic activation and detoxification of polycyclic hydrocarbons and other chemical carcinogens.

This section studies the molecular events of malignant transformation induced by chemical carcinogens, in particular those of the polycyclic hydrocarbon class. The aim is to understand the enzymatic conversion of carcinogens to either detoxification forms or to the active carcinogenic metabolite. During evolution, humans have been exposed to foreign chemical compounds (including carcinogens) and have developed systems for their detoxification and elimination. These systems primarily involve microsomal cytochrome P-450 mixed-function oxygenases, but also include epoxide hydratases and conjugating enzymes. The vast majority of foreign compounds are processed by these enzyme systems. The mixed-function oxygenases are influenced by a variety of enviromental factors such as drugs, pesticides or carcinogens, and are influenced by the nutritional and hormonal state of the animal. The age, sex and genetic makeup also determine enzyme activity. Work in this laboratory provided the key studies which showed this enzyme system to be responsible for the activation of procarcinogens to what may be their ultimate carcinogenic form.

A primary goal is to define the enzymatic mechanism by which polycyclic hydrocarbons (PCH) are activated either to carcinogenic forms or to detoxified products. As these enzymes are characterized and as sensitive methods are developed for their assay, it may be possible to characterize an individual's enzymatic makeup with respect to carcinogen metabolism and to understand the relationship between this metabolism and individual susceptibility to PCH carcinogenesis. The known sequence of PCH metabolism is as follows: The first step is oxygenation by the microsomal mixed-function oxygenases. Aryl hydrocarbon hydroxylase (AHH) activity is one indicator of this step. Very sensitive methods have been developed for AHH activity which are applicable to human tissues. Secondly, epoxides are hydrated to dihydrodiols by epoxide hydratase. The epoxides are also metabolized by glutathione S-transferases. The phenols and dihydrodiols are conjugated to UDP glucuronic acid or as sulfates. Sensitive assays for each of these systems, as well as a method for total metabolite analyses by high pressure liquid chromatography (HPLC), have been developed.

The approach is to identify and fully characterize the enzymes responsible for carcinogen activation and metabolism. In addition, we seek to understand the molecular biology and regulation of this system both at the genetic and epigenetic levels. This knowledge may be important for understanding the role of biochemicals individually in carcinogenesis. We plan to assess the types and amounts of these enzymes in human populations using metabolic, immunological and molecular biological approaches. We will carry out multileveled investigations of carcinogen metabolizing enzyme systems, continuing our use of high pressure liquid chromotography to study carcinogen metabolites, using monoclonal antibodies and enzyme inhibitors to study the properties of the enzymes and using recombinant DNA and other molecular biological techniques to study the stucture and regulation of the genes for the enzymes of carcinogen metabolizing systems.

Earlier, we introduced the use of high pressure liquid chromatography for the analysis of polycyclic aromatic hydrocarbon metabolism. We are continuing this development in an attempt to improve the separation efficiency and reproducibility of polycyclic aromatic hydrocarbon metabolism.

We have found that the addition of anti-oxidants prevents oxidation of low levels of metabolites and we have used this improvement to analyze low levels of metabolism in human cells.

We have taken a completely new and very promising approach to the study of biochemical individuality in human carcinogenesis. We are using monoclonal antibodies as tools to probe the types and profiles of human carcinogen metabolizing enzymes in different individuals. We have now successfully prepared monoclonal antibodies to two rabbit liver cytochromes P-450, designated LM_2 and LM_4. We have also prepared monoclonal antibodies to both the MC-type and PB-type cytochromes P-450 from rat liver. The monoclonal antibody for MC-type rat liver cytochrome P-450 also strongly inhibits the AHH activity of placental microsomes from smoking women.

We have adopted recombinant DNA and related molecular biological techniques to assess, with increased precision, the molecular mechanisms of regulation of P-450 gene expression, the multiplicity of P-450s and the structural-functional relationships among the P-450s.

This work also has potential toward increasing our understanding of biochemical individuality in human carcinogenesis. We have successfully constructed recombinant bacterial plasmids carrying cDNA complementary to the mRNA for methylcholanthrene (MC) type cytochrome P-450 from rat. We are now isolating recombinant plasmids carrying phenobarbital-type cytochrome P-450 mRNA sequences. We have already used the MC-P-450 mRNA clone and in vitro translation to demonstrate that MC-P-450 mRNA is four-fold elevated in liver from MC-treated rats and that there are two size classes of mRNA which code for MC-induced cytochrome P-450. The cDNA clones will also be used to clone the native genes for MC- and PB-type cytochromes P-450 as a first step toward analysis of the structure of the cytochrome P-450 genes.

Another key enzyme involved in carcinogen metabolism is epoxide hydratase. A rather important question has been the role of this enzyme in the detoxification of the active carcinogenic forms of BP. We have found that in a variety of conditions the diol epoxides are susceptible to the enzymatic action of the epoxide hydratases, thus establishing a role for this enzyme in carcinogen deactivation.

Human lymphocytes and monocytes have been shown to exhibit mixed-function oxidase activity, a property which in many tissues and species is responsible for the activation of polycyclic hydrocarbon carcinogens. The levels of this activity vary among individuals and may be enhanced by culturing the cells with various inducers. In rat and rabbit tissues, cytochrome P-450 is present in multiple forms and these forms differ in their substrate specificities. Multiple forms of cytochrome P-450 have not yet clearly been demonstrated in human tissues. We have made use of a new and distinctive AHH inhibitor, 1-Maackiain, in conjunction with the known inhibitor 7, 8-benzoflavone, to demonstrate that not only are there two distinct forms of cytochrome P-450 in rat liver, lung and kidney, but, also that the form of cytochrome P-450 induced in humans by cigarette smoke is quite distinct from that found in normal liver. Thus, there are, in fact, multiple cytochromes P-450 in human tissues.

Nucleic Acids Section - Studies (1) interaction of chemical carcinogens and their analogs with nucleic acids and the action of cellular processes regulating nucleic acid metabolism; and (2) the effect of these agents on the subcellular organization of nucleic acid macromolecules.

The primary areas of research in this section are: 1) an investigation of the mechanism of DNA repair in normal and malignant cells in order to understand the relationship of DNA damage and its repair to human cancer; 2) determination of the structural-functional relationships of DNA modified with chemical carcinogens; and 3) the possible role that misreading of aminoacyl-tRNAs during protein synthesis may have in carcinogenesis and changes in aminoacyl-tRNA (anti-codon: codon and tRNA: aminoacyl-tRNA synthetase interactions).

A group of 17 of the 88 human tumor cell strains has been identified as deficient in repairing methylation damage. Thirty-eight strains of normal human fibroblasts were proficient in such repair, including two fibroblast strains prepared from people whose tumors gave rise to repair-deficient strains. Such repair-deficient cells were killed by low concentrations of alkylating agents, including chemotherapeutic agents, repaired alkylated infecting viruses poorly and failed to remove O^6-methylguanine from their DNA after methylation treatment. Strains both deficient and proficient in the repair of methylation damage were found to have relaxed DNA after methylation treatment, but only the repair-proficient strains could restore their DNA to normal conformation. One repair-deficient strain was also deficient in post-methylation DNA relaxation, and two repair-proficient strains, showing a slow post-methylation restoration of DNA conformation, were somewhat sensitive to methylation killing.

Novobiocin and naladixic acid were found to block DNA-relaxation reversibly when cells were treated with UV or MNNG. Inhibition of DNA polymerases blocked a later step in repair, decreasing the rate of regeneration of normal DNA conformation. Human tumor cells were found less able than human diploid fibroblasts to discriminate against stably incorporating the nucleoside analog bromodeoxyuridine (BUDR) into their DNA. Adenoviruses grown in the presence of BUDR in normal cells are killed less by BUDR than if grown in the same conditions, but in tumor cells. Human tumor cells and human diploid fibroblasts were shown to use distinguishable mechanisms for synthesizing their DNA.

Attempts to establish sensitive methods for detecting structural and biologically significant modifications of a DNA plasmid using BPDE-1 have led to the findings that at least 2/3 of the potentially lethal damage caused in the plasmid by BPDE-1 are repaired by the UVR repair system of E. coli, and that BPDE-1 mutagenized the plasmid by causing insertion (A:T pair) and deletion (G:C pair) frame shifts.

A method utilizing two-dimensional gel electrophoresis, previously developed in this section, was employed to study the effects of partial dehydration due to organic solvents on the conformation of closed circular DNAs. The results permitted the calculation of thermodynamic parameters of DNA unwinding as with the dehydration. Further, it was determined that hydroxylation of B(a)P at the 7 and 8 positions facilitates intercalation with DNA.

In studies on the activities of isoacceptor aa-tRNAs, it has been found that phe-tRNA containing Wye base and phe-tRNA without Wye base are transferred to protein at non-identical rates. A particular undermodified Lys-tRNA, Lys-$tRNA_4$, does not transfer its lysine into AAG coded sites as frequently as other lysine isoacceptors with the same coding properties and further, misreads AAA with a relatively high frequency.

Protein Section - Studies (1) protein-nucleic interactions which may regulate or coordinate the production of cellular proteins, (2) specific alterations in DNA sequences which may modify control functions or lead to altered levels of regulatory proteins, (3) mechanisms which modify genetic information in DNA by post-transcriptional processing of RNA.

A new method for estimation of chain lengths of ribonucleic acids is under development. The method involves partial heat degradation of the RNA under study and measurements of the remaining and degraded molecules. From similar measurements on reference RNAs, the chain lengths may be deduced. In addition, computer simulation permits computation of the relation between chain length and electrophoretic mobility for use in electrophoretic analysis of polydisperse samples. Heterogeneous nuclear RNA from HeLa cells has been studied by these techniques. The number-average RNA chain is approximately 5,000 nucleotides long; the weight-average is about twice as great.

Measurements of the amount of steady-state hnRNA and parameters characterizing its size distribution may be of value in understanding the biological properties of malignant cells. The populations of hnRNA found in several transformed and normal 3T3 cells were compared, using three different labeling times, and improved electropheretic and computational methods. The hnRNA populations from normal and malignant cells were very similar.

The highly phosphorylated glycoprotein found in human and murine milk, whose discovery was reported in the last report, has been isolated in small amounts. Quantitative analysis of the human protein indicated the presence of 10 moles of phosphorus per mole of protein; there was less than one percent sialic acid. Although the composition of milk has been intensively examined, it appears that techniques developed in this project have permitted the discovery of a new protein constituent.

Antibodies specific to histones and to non-histone chromosomal proteins have been used to study the structure-function relation of defined proteins in chromatin and chromosomes. A sensitive and convenient enzyme linked immunoassay for analysis of these proteins has been developed. The antibody levels against chromosomal proteins in 50 patients suffering from autoimmune diseases have been determined. Antibodies to the globular region of histone H_5 have been elicited. Microinjection of antibodies to histone H_2B and non-histone HMG-1 into the nuclei of oocytes of Pleurodeles waltlii bring about a specfic retraction of the transcriptional loops in the lampbrush chromosomes present in these oocytes. Microinjection of antibody fragments into the nucleus and cytoplasm of somatic cells indicate that $F(ab)_2$ does, but IgG does not, traverse the nuclear membrane. Histone antigenic determinants are masked in the transcriptionally active regions of polytene chromosomes.

The interaction of a chemical carcinogen with the genome of a target cell is studied by 1) examining the binding of benzo(a)pyrene to SV40 minichromosomes, 2) examining the binding of benzo(a)pyrene to polytene chromosomes, and 3) using antibodies produced by benzo(a)pyrene modified DNA. The results indicate that 1) the packing of the cellular DNA into the nucleosomal conformation does not significantly affect the binding of the carcinogen to DNA, 2) the *in vivo* incorporation of benzo(a)pyrene adducts into polytene chromosomes do not reveal "hot spots" for carcinogen binding, and 3) the binding of BPDE-1 to the genome can be visualized by immunofluorescence and by immunoelectron microscopy.

A system has been developed to assay DNA sequence changes which arise in cells as a result of covalent modification of the DNA by chemical carcinogens. The program involves modification of a specific DNA fragment with the active form of benzo(a)-pyrene, the 7, 8-diol, 9, 10-epoxide, followed by ligation of the fragment into an appropriate plasmid vector. The ligation products are introduced into cells, replicated and the progeny DNA molecules harvested and screened for changes in the sequence of the modified fragment. This protocol is designed to measure forward mutations and avoid the selective constraints of the standard back mutation mutagenesis assays. In addition, it will permit the distinction between sequence changes which arise directly from DNA modification and those which occur from error-prone replication induced by DNA damage at distal sites. Although the amplification and assay of mutagenic events occur in bacteria, appropriate vectors can be used for the same types of experiments in human cells followed by analysis in bacteria.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04496-04 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

In Situ Organization of Chromosomal Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Bustin	Visiting Scientist	LMC	NCI
OTHER:	P. D. Kurth	Staff Fellow	LMC	NCI
	L. Einck	Guest Worker	LMC	NCI

COOPERATING UNITS (if any)

Division of Membrane Biology, German Cancer Center, Heidelberg, Germany
Department of Biophysics, Kings College, University of London, London, England

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.9	PROFESSIONAL: 2.5	OTHER: 1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies specific to histones and to non-histone chromosomal proteins have been used to study the structure-function relation of defined proteins in chromatin and chromosomes. A sensitive and convenient enzyme linked immunoassay for analysis of these proteins has been developed. The antibody levels against chromosomal proteins in 50 patients suffering from autoimmune diseases has been determined. Antibodies to the globular region of histone H_5 have been elicited. Microinjection of antibodies to histone H_2B and non-histone HMG-1 into the nuclei of oocytes of Pleurodeles waltlii bring about a specific retraction of the transcriptional loops in the lampbrush chromosomes present in these oocytes. Microinjection of antibody fragments into the nucleus and cytoplasm of somatic cells indicate that $F(ab)_2$ does, but IgG does not, transverse the nuclear membrane. Histone antigenic determinants are masked in the transcriptionally active regions of polytene chromosomes.

Project Description

Objectives: To understand the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes. To explore the possibility that neoplastic transformation is associated with defined alterations in either the type or the organization of chromosomal components.

Background Information and Research Strategy: The chromatin fiber, which is the backbone of chromatin and chromosomes, is a dynamic structure. Thus, it is difficult to determine the structure-function relation of defined chromosomal components. This question was approached by purifying chromosomal proteins, eliciting specific antibodies to these and using the antibodies as probes for studying the *in situ* arrangement of the defined chromosomal components at various stages of chromatin organization. So far, it was possible to purify the antigens, elicit antibodies, and adapt and develop various immunochemical techniques for detecting and quantifying the binding of specific antibodies to chromatin and chromosomes. Immunoelectron microscopy and immunofluorescent techniques are used to study the organization of histones and defined non-histone chromosomal proteins in the interphase, transcribing and replicating chromatin fiber and in metaphase and polytene chromsomes.

In the past year, the research effort was concentrated in the following areas: 1) development of new enzyme linked immunassays for chromosomal proteins; 2) development of new antisera and determination of their specificity; 3) studies on the *in vitro* and *in vivo* transcripts of chromosomal proteins HMG-1 and HMG-2; 4) studies on the organization of chromosomal proteins in transcribed regions of polytene chromosomes; and 5) introduction of functional antibodies and antibody fragments into living nuclei and cells.

Methods Employed: Enzyme linked immunoassay, solid phase radioimmune assay, immunofluoresence, DBM paper transfer, immunoprecipitation, translation of mRNA, microinjection.

Major Findings: 1) New immunological assays for chromosomal components.

Sensitive, quantitative and convenient immunological assays are prerequisites for a variety of immunological studies on chromosomal proteins. These techniques are of specific importance for chromosomal proteins, many of which do not have an assayable function. In the previous report, we described the development of a sensitive solid phase radioimmune assay and pointed out that the test can potentially be converted to an enzyme-linked immunoassay. This has been accomplished in our laboratory. The enzyme-linked immunoassay (ELISA) is suitable for detection and quantitation of all histone fractions, non-histone proteins HMG-1, HMG-2 and HMG-17 and specific histone fragments. It can reliably detect as little as 100 pg of antigen in 100 ul using sera dilutions of over 1:200,000. Furthermore, the assay is semi-automatic and eliminates the use of radioactive materials. Antibodies coupled to peroxidase or to alkaline phosphatase are suitable for the assay. The microtiter plates, to which the antigen is bound, can be reused after washing the antibodies off by 6M Guanidinium hydrochloride.

Sera from patients suffering from certain autoimmune diseases contain antibodies specific to nuclear components. The ELISA was used to screen such sera from 54 patients for the content of antibodies against chromosomal proteins H_2A, H_2B, H_3, H_4, HMG-1, HMG-2 and HMG-17. While all the patients have antibodies against DNA, their content of antibodies to chromosomal proteins showed a dependency on the type of autoimmune disease. None of the 14 patients suffering from rheumatoid arthritis, and only 2 of the 11 patients suffering from mixed connective tissue diseases, had antibodies against the proteins tested. In contrast, 15 of the 29 patients diagnosed as suffering from systemic lupus erythematosus contained such antibodies. Eleven patients had antibodies directed against H_2A and H_2B and only 3 had a very weak response to H_4 and H_3. Nine patients had a strong response to HMG-17, but only 3 had a weak response to HMG-1 and HMG-2. The results suggest that in the autoimmune diseases the intact nucleosome serves as an immunogen. It may be possible to use peripheral lymphocytes from selected patients to produce human monoclonal antibodies of desired specificity.

2) Development of new antisera and determination of their specificity.

Antisera to the globular region of histone H_5 have been elicited and their specificity determined by ELISA, and by the DBM paper transfer technique. The antibodies cross-react with intact H_5 histone, but not with histone H_1. Histone H_1 is a group of proteins (about 5 molecular species per tissue) which is the only histone that displays some degree of tissue specificity. It is probably involved in determining and maintaining the higher order structure of chromatin. During maturation of avian erythrocytes, the H_1 is replaced by H_5 histone which binds to chromatin much stronger than H_1. Preliminary results suggest that a mammalian analogue of the avian H_5 accumulates in cells which have stopped dividing. The availability of the antibody to the globular region of H_5 will enable us to study the structure and function of this protein in a novel way.

3) Studies on the in vivo and in vitro biosynthesis of chromosomal protein HMG-1.

Chromosomal protein HMG-1 is located at the periphery of the nucleosomes, probably attached to internucleosomal linker region. Preliminary results suggest that this protein, or closely related molecular species, may shuttle between the nucleus and cytoplasm. We have investigated how many cellular molecular species there are and whether this protein in synthesized via precursors. Immunoprecipitation of nuclear and cytoplasmic extract obtained from HeLa, KD, and green monkey cells, which were radioactively labeled with ^{3}H-lysine, revealed that the antibody interacts only with HMG-1 and HMG-2. No precursors or immunologically related molecular species were detected. A similar result was obtained using a cell-free translation system. Total RNA was extracted from HeLa, rat liver and rat thymus. The RNA from rat thymus was fractionated into poly A^+ and poly A^- RNA. The poly A^+ fraction coded for HMG-1 and HMG-2 while the poly A^- did not. This indicates that the processing of the primary transcript of this non-histone protein is different from that of the histones. Future experiments are designed to study further the function of this protein.

4) The organization of proteins in transcribed regions of polytene chromosomes.

Transcription can be easily visualized in polytene chromosomes by conventional light microscopy and by autoradiography. We have found that incubation of salivary glands from chironomus thummi with solutions of various ionic strength stimulates transcription as evidenced by incorporation of ^{3}H-uridine followed by autoradiography. The exposure of antigenic determinants in histone H_3 and the non-histone protein HMG-1 in these transcriptionally active chromosomes was assessed by indirect immunofluorescence. We find that increased transcriptional activity brings abount a masking of antigenic determinants of histone H_3 but not of protein HMG-1. Counterstaining with ethidium bromide indicates that the reduction in the level of immunofluorescence was not due to dilution of the chromatin fiber density over a wider area. Treatment with acetic acid in the absence of chromosome cross-linking brings about an exposure of the histone antigenic determinants in the puffed chromsomes. We suggest that transcriptional activity in the chromosomal loci is associated with an influx of proteins to the transcribed regions which results in the masking of antigen determinants. In the future, we plan to further elucidate the structural rearrangements associated with gene activation.

5) Introduction of functional antibodies and antibody fragments into the cytoplasm and nuclei of living cells.

Introduction of functional antibodies into living cells potentially may lead to the understanding of the in vivo cellular role of the respective antigen. Antibodies to chromosomal proteins H_2B and HMG-1 were microinjected into the oocyte nuclei of the salamander Pleurodeles waltlii. These oocytes are a convenient experimental system because they are large and contain lampbrush chromsomes. These chromsomes contain several thousand highly active genes which can be visualized in the transcribing process both by electron and by conventional light microscopy. Both type of antibodies bound to the lampbrush chromosomes, as evidenced by immunofluoresence. Furthermore, DBM transfer blots revealed that the chromsomes contain a protein cross-reacting with HMG-1. Microinjection brings about the retraction of the transcriptional loops and an overall shortening of the chromosomes. The effect was similar to that observed when antibodies to RNA polymerases were microinjected or when the oocytes were treated with the RNA polymerases inhibitor α-amatin. No such effects were seen when antibody to tubulin, actin, nucleoplasmin, and normal IgG were microinjected. Detailed electron microscopic studies on the treated chromosomes revealed that the ribosomal transcripts were significantly less affected than the rest of the transcripts. In parallel to the microinjection into oocytes, we have introduced into the laboratory the microinjection techniques of Graessmann which allows the use of somatic mammalian cells. IgG and antibodies, $F(ab)_2$ fragments and chromosomal proteins were fluorescinated or rhodaminated and then microinjected into either the nucleus or the cytoplasm of KD cells. The results indicate the $F(ab)_2$ fragments do, and the IgG do not, transverse the nuclear membrane. These results indicate that for in vivo localization and functional studies using immunochemical techniques it is necessary to use $F(ab)_2$ fragments. The results are also applicable to studies where antibodies are used to deliver drugs that inhibit DNA synthesis into special cells.

Significance to Biomedical Research and the Program of the Institute: Understanding the mechanism of gene regulation and its relation to neoplasia requires knowledge of the structure of chromatin and chromosomes. The approach developed in this laboratory is presently the only approach in which specific probes for well-defined purified chromosomal components are used to study the organization of these components in intact chromatin and chromosomes. As such, a unique opportunity has developed whereby certain structural aspects of these nucleoproteins can be visualized and directly related to functional stages of the genome. The immunological techniques developed for the study of the *in situ* organization of proteins in chromatin and chromosomes are applicable to studies on damage and repair in the genome as a result of binding of carcinogens or X-ray and UV exposure.

Proposed Course: Studies devoted to understanding the structure function relation of defined chromosomal components will be continued. In the forthcoming year, we will concentrate our efforts on 1) utilizing the microinjection technique to understand the *in vivo* function of defined chromosomal proteins; 2) elucidating biosynthetic aspects of chromosomal protein HMG-1; 3) developing new, defined, serological reagents including the possibility of monoclonal antibodies; and 4) mapping chromosomal proteins in chromatin and chromosomes.

Publications:

Bustin, M., and Feldman, R. J.: Chromatin subunit interactions studies by computer graphics. *Europ J. Cell Biol.* 22: 75, 1980.

Isackson, P. J., Bidney, D. L., Reeck, A. R., Neihart, N. K., and Bustin, M.: High mobility group chromosomal protein isolated from nuclei and cytosol of HTC cells are similar. *Biochemistry* 19: 4466-4471, 1980.

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Kurth, P. D., and Bustin, M.: Localization of chromosome protein HMG-1 in polytene chromosomes of chironomus thummi. *J. Cell Biol.* 89: 70-77 1981

Tahourdin, C. S. M., and Neihart, N. K., Isenberg, I. and Bustin, M.: Immunchemical detection of chromosomal protein HMG-17 in chromatin subunits. *Biochemistry* 20: 910-915, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04516-05 LMC

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular and Molecular Effects of Psoralen Plus Ultraviolet Light

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. H. Kraemer	Research Scientist	LMC	NCI
OTHER:	R.E. Tarone	Mathematical Statistician	B	NCI
	W.L. Levis	Senior Investigator	D	NCI
	K. Kohn	Chief	LMPH	NCI
	D.G. Glaubiger	Senior Investigator	PO	NCI
	P. Pizzo	Senior Investigator	PO	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	0.6	1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Psoralen plus long wavelength ultraviolet radiation(UV-A) is being investigated as a model system for clinically relevant photochemical carcinogenesis. Used experimentally for treatment of psoriasis and mycosis fungoides, psoralen plus UV-A has been found to be mutagenic in bacteria and carcinogenic in mice and humans. We have developed an in vitro assay to measure the effects of UV-A mediated psoralen-DNA binding on human lymphoid cells. Parameters monitored include the rate of DNA synthesis, induction of DNA-psoralen cross-links, induction of sister chromatid exchanges, alterations in the rate of cell proliferation and survival, and in immune reactivity. Presently we are investigating whether lymphoid cells from patients with cancer-prone genetic diseases have increased sensitivity to psoralen plus UV-A induced cell killing. These studies are aimed at understanding the mechanism of cell damage induced by psoralen plus UV-A so as to minimize the toxicity to human cells during therapy.

Project Description

Objectives: Humans are exposed to chemicals which may interact with ultraviolet radiation to become carcinogenic. 8-methoxypsoralen (8-MOP), a compound which is found in many plants, plus high intensity long wavelength ultraviolet radiation (UV-A) is being used experimentally to induce remissions in psoriasis and in mycosis fungoides. The combination of 8-MOP plus UV-A produces DNA-8-MOP binding and has been shown to induce mutations in bacteria and in mammalian cells and to cause skin cancer in mice and humans. We are attempting to develop an in vitro model system to assess clinically relevant photochemical carcinogenesis.

Methods Employed and Major Findings: We previously demonstrated that circulating lymphoid cells of some psoriasis patients receiving 8-MOP plus UV-A therapy had a significant reduction in DNA synthesis. We have developed an in vitro assay system to approximate some of the conditions of 8-MOP plus UV-A on human lymphoid cells in vivo during therapy. The assay has been used with fresh lymphocytes and with long-term lymphoblastoid cell lines. These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells.

We have developed a simple microtiter assay to measure lymphoblastoid cell survival after treatment with 8-MOP plus UV-A or other DNA damaging agents and have automated the end-point analysis of this assay.

In the lymphoblastoid cells, as much as a 50% inhibition of DNA synthesis following 8-MOP plus UV-A treatment was associated with 100% survival. Greater inhibition of DNA synthesis resulted in an exponential decrease in cell survival. Similarly, measurements of 8-MOP-DNA cross-linking by the alkaline elution technique revealed a dose dependent increase in cross-link induction above a threshold of approximately 50% inhibition of DNA synthesis. The formation of cross-links was also correlated with decreased cell survival. Thus, DNA-8-MOP interstrand cross-links may be responsible for inhibition of DNA synthesis and cell killing.

8-MOP plus UV-A treatment of lymphocytes or lymphoblastoid cells in vitro resulted in approximately a doubling in the number of sister chromatid exchanges per metaphase. Further increases in 8-MOP plus UV-A were toxic. Thus, it is likely that the doses of 8-MOP plus UV-A received by patients' lymphocytes are too low to permit routine detection of increased sister chromatid exchanges.

Mixed leukocyte reactivity of fresh human leukocytes was found to be inhibited in a dose dependent manner by 8-MOP plus UV-A in vitro, stimulator and responder functions were both inhibited. This inhibition of immune reactivity may be related to in vivo carcinogenesis.

Cancer patients who develop cutaneous infection with varicella-zoster virus may suffer increased morbidity and mortality. 8-MOP plus UV-A has been demonstrated to decrease herpes virus multiplication in mice. In collaboration with investigators in the Pediatric Oncology Branch, we are studying the therapeutic efficacy of 8-MOP plus UV-A for cancer patients with herpes zoster infection. This laboratory provides dermatologic expertize and phototherapy equipment. Patients have recently begun treatment under a controlled protocol.

Significance to Biomedical Research and the Program of the Institute: These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells.

Proposed Course: Lymphoblastoid cell lines from patients with cancer-prone genetic disease are being examined for evidence of hypersensitivity to psoralen plus UV-A induced killing. This may indicate populations who are at increased risk of toxicity from photochemotherapy.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04517-05 LMC

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

DNA Repair In Human Cancer-Prone Genetic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.H. Kraemer	Research Scientist	LMC	NCI
OTHER:	M.H. Greene	Clinical Epidemiologist	EEB	NCI
	J. Fagan	Staff Fellow	LMC	NCI
	J. Bader	Clinical Investigator	CEB	NCI
	H.V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human cancer-prone genetic diseases are being studied in order to identify groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting to determine the clinical consequences as well as the molecular basis of their cellular hypersensitivity. Patients with xeroderma pigmentosum (XP) and ataxia telangiectasia, diseases with ultraviolet and X-ray sensitivity, respectively, and with familial malignant melanoma (dysplastic nevus syndrome) and neurofibromatosis, diseases with no documented environmental sensitivity, are being studied. Detailed examinations of the clinical features of affected individuals are being made. A registry of XP patients is being established. Cultures of skin and blood are being established and the effects on cell survival, DNA repair, and chromosome abnormalities after treatment with DNA damaging agents (ultraviolet, bleomycin, or psoralen plus long wavelength ultraviolet) are being examined. These studies may identify persons with increased risk of cancer and suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

Project Description

Objectives: Human cancer-prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting (1) to correlate such sensitivity with clinical abnormalities, (2) to determine if there is genetic diversity within such groups, and (3) to understand the molecular basis of their cellular hypersensitivity.

Methods Employed: Patients are examined with particular emphasis on cutaneous abnormalities, and cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory experimentation. Patients with xeroderma pigmentosum (XP), ataxia telangiectasia (AT), familial malignant malanoma (dysplastic nevus syndrome, DNS) and neurofibromatosis (NF) have been studied.

Major Findings: XP, is an autosomal recessive cancer-prone disease with clinical ultraviolet (UV) sensitivity, accompanied by cutaneous and neurological abnormalities. Cultured cells from XP patients have cellular UV sensitivity and defective DNA repair. We have compiled the most comprehensive review of the world literature, to date, on XP including both clinical and laboratory observations. Selected XP patients with unusual clinical features are being studied for cellular UV sensitivity and abnormal DNA repair. We are attempting to clone the DNA genes which will correct the ultraviolet hypersensitivity in XP lymphoblastoid cell lines. A registry of XP patients is being established. The question of the possible role of environmental mutagens in internal neoplasia is being approached by attempting to determine if XP patients have increased frequency of internal as well as cutaneous neoplasms.

AT, an autosomal recessive cancer-prone disease with cutaneous, neurological and immunological abnormalities has X-ray sensitivity. We are studying the ability of cultured cells from AT patients and their parents to survive DNA damage induced by the chemotherapeutic agent, bleomycin. We have found that AT homozygous and heterozygous lymphoblastoid cell lines have a range of sensitivity to killing by bleomycin. Further, this agent induces an abnormally large increase in chromosome breakage (but not in sister chromatid exchanges) in AT lymphoblastoid cells. Studies of the specificity of the AT cellular defect are in progress using UV and psoralen as other DNA damaging agents.

A newly recognized clinical disease, familial malignant melanoma with a characteristic precursor legion, the dysplastic nevus, is being examined in collaboration with the Environmental Epidemiology Branch, NCI. This laboratory is contributing dermatological expertize in the clinical definition of the syndrome in a study of more than 300 family members. Lymphoblastoid cell lines from selected patients are being examined for evidence of sensitivity to DNA agents, and possible DNA repair defects.

NF, an autosomal dominant disease with characteristic cutaneous pigmentary lesions and multiple nervous system tumors, is being examined in collaboration with the Clinical Epidemiology Branch, NCI. Selected patients with different forms of NF are examined clinically and cultures of skin and blood are established. This laboratory is providing dermatological advice and expertise in examination of the patients and will study cultured cells for evidence of sensitivity to DNA damaging agents.

Patients with psoriasis, a benign disease with a strong genetic component, have rapid, but non-neoplastic, proliferation of lesional epidermal cells. We are studying the level of arylhydrocarbon hydroxylase (AHH) activity, an important enzyme in carcinogen metabolism, in lymphocytes and monocytes from psoriasis patients in collaboration with the Metabolic Control Section, LMC. This laboratory is examining the patients clinically and obtaining blood for analysis. Age-related changes in AHH activity were found in monocytes and lymphocytes. In addition, topical therapy with steroids and/or tar appeared to induce AHH activity in circulating leukocytes.

Significance to Biomedical Research and the Program of the Institute: These studies may identify persons with increased risk of cancer and suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

Proposed Course: This project will be continued along the lines indicated above.

Publications:

Kraemer, K.H. Xeroderma pigmentosum: A prototype disease of environmental-genetic interaction. Arch. Dermatol. 116: 541-542, 1980.

Kraemer, K.H. Xeroderma Pigmentosum. In Demis, D.J., Dobson, R.L. and McGuire, J. (Eds): Clinical Dermatology. 1980, New York, Harper and Row Publishers. Vol. 4, Unit 19-7, pp 1-33

Kraemer, K.H.: Oculo-cutaneous and internal neoplasms in xeroderma pigmentosum: Implications for theories of carcinogenesis. In Pullman, C., Ts'O, P.O. and Gelboin, H.V., (Eds). Carcinogenesis: Fundamental Mechanisms and Environmental Effects. 1980, Dordrecht, Reidel, pp. 503-507.

Kraemer, K.H., Buchanan, J.K., and Stinson, S.F.: Semi-automated autoradiographic grain counting instruments for measurement of DNA repair. In Vitro 16: 609-615, 1980.

Smith, P.J., Paterson, M.C., and Kraemer, K.H.: In vitro radiosensitivity in a patient with dermatomyositis and cancer. Lancet 1: 216-217, 1981.

Kraemer, K.H.: Cancer-prone genodermatoses and DNA repair. Progress in Dermatology (In Press)

Kraemer, K.H., and Robbins, J.H.: Xeroderma pigmentosum. In Maddin, S., (Ed): Current Therapy in Dermatology. Philadelphia, W.B. Saunders Co. (In Press)

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04518-04 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

AA-tRNA Populations in Mammalian Cells and role of Isoacceptors in Protein Synthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Hatfield	Research Biologist	LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Relative utilization of isoacceptor aa-tRNAs in protein synthesis have demonstrated variations in the incorporation of the corresponding amino acid into specific sites in protein even though the isoacceptors have the same codon recognition properties. Three minor Ser-tRNAs, two of which recognize specificially the nonsense codon UGA, have been further examined. The levels of isoacceptors which recognize specific codons have been determined in human and rabbit reticulocytes and a significant correlation exists between these levels and the corresponding codewords in globin mRNA from the respective tissues. An examination of the tRNA population from mammary tissues has been undertaken.

Project Description

Objectives: The objectives are: 1) to determine the effect of specific base modifications in isoacceptor aa-tRNAs on protein synthesis and whether misreading genetic code words by isoacceptor aa-tRNAs during protein synthesis may be involved in the expression of cancer; 2) to determine the cellular function of three minor Ser-tRNAs, two of which recognize the nonsense codon UGA ($\text{Ser-tRNA}_{\text{UGA}}$); 3) to examine the levels of isoacceptor aa-tRNAs which recognize specific codons in human and rabbit reticulocytes and determine if a correlation exists between these levels and the frequency of usage of the corresponding codons in globin mRNA from the respective tissue. and 4) to examine the aa-tRNA population of mammary tissue in order to determine if specific isoacceptors may be enriched during lactation.

Methods Employed: Isoacceptors of tRNA^{Lys} and tRNA^{Phe} were purified extensively by standard chromatographic techniques and aminoacylated with the corresponding labeled amino acid. The labeled aa-tRNAs were then added to rabbit reticulocyte lysates and to wheat germ extracts. The relative rates of incorporation of the corresponding amino acid from each isoacceptor into protein and into specific sites of protein were then determined by chromotography of the aa-tRNA remaining after protein synthesis and by digestion of the protein product with trypsin and examination of the resulting peptides. Three minor Ser-tRNAs, two of which recognize specifically the nonsense codon UGA, have been isolated from bovine liver by a simple procedure which permits the processing of large quantities of tissue. This procedure entails making an extract of bovine liver followed by the attachment of the more hydrophopic tRNAs to DEAE-cellulose, subsequent removal of tRNA-Ser from DEAE cellulose and attachment to and removal from BD-cellulose.

The levels of isoacceptor aa-tRNAs to which codon assignments have been made in rabbit (see Hatfield, Matthews and Rice, *Biochim. Biophys.Acta* 564: 414, 1979) and in human reticulocytes (Hatfield, Varricchio, Rice and Forget, unpublished work) have been determined by standard chromatographic techniques and compared to the frequency of usage of the corresponding codons in rabbit and human globin mRNA. (Sequences of α- and β-globin mRNA from rabbit and human reticulocytes have been determined in other laboratories.)

Transfer RNA has been isolated from fresh bovine mammary tissue by standard techniques. Our focus of attention thus far has been on the three minor Seryl-tRNAs characterized from bovine liver (see above), since at least one of these isoacceptors has been reported to form phosphoseryl-tRNA in mammary tissues (see S. Sharpe and T. Stewart, *Nucleic Acids Res*. 4: 2123, 1977). Techniques are presently being devised to separate the minor Seryl-tRNAs from other mammary aa-tRNAs by a combination of procedures, developed in other laboratories, using polyacrylamide gel electrophoresis.

Major Findings: We have previously shown that Phe-tRNA which contains Wye base (from normal cells) and Phe-tRNAs which lack Wye base (from FrNTD and neuroblastoma cells) transfer phenylalanine to protein at similar, but not identical rates. A careful examination of the rates of deacylation and of incorporation of phenylalanine into protein in wheat germ extracts programmed with globin mRNA has demonstrated that the different rates of incorporation are correlated with the rate of deacylation of each Phe-tRNA, i.e., Phe-tRNA from neuroblastoma cells which transfers phenylalanine to protein more extensively than from other Phe-tRNAs is deacylated more slowly (see reference 2 below). (Studies on Phe-tRNA with and without Wye base were a collaboration with Dr. J. F. Mushinski, LOBGY, NCI).

Specific site incorporation of an undermodified isoacceptor of Lys-tRNA (designated Lys-tRNA^4), which we have shown to be utilized less frequently in protein synthesis than other lysine isoacceptors with the same codon recognition properties (see reference 1 below), has shown (1) that LystRNA$_4$ does not transfer its amino acid into AAG coded sites as frequently as other lysine isoacceptors which have the same codon recognition properties (e.g., Lys-tRNA$_{1,2}$); and (2) that Lys-tRNA$_4$ is capable of misrecognizing AAA codewords more frequently than Lys-tRNA$_{1,2}$. (This study has been a collaboration with Dr. David Smith of Northwestern University.)

A significant correlation between the levels of isoacceptors which recognize specific codons in rabbit and human reticulocytes and the corresponding codon frequency in globin mRNAs from the respective tissues was found.

Minor Ser-tRNAs, which have been isolated from bovine liver, have previously been resolved into three isoacceptors, two of which recognize, specifically, the nonsense codon, UGA, and the third which does not recognize any serine or nonsense codon. Sequences of the isoacceptors which recognize UGA have been determined by Drs. A. Diamond and B. Dudock of Stony Brook University, and one of these isoacceptors is capable of suppressing UGA in protein synthesis.

The minor Ser-tRNAs described above are enriched in bovine mammary tissue. We are in the process of separating these Ser-tRNAs from bulk mammary tRNA by polyacrylamide gel electrophoresis. Once this is accomplished, we plan to obtain labeled tRNA from mammary tissue in culture and to determine the effects of specific hormone treatment on the levels of the minor Seryl-tRNAs as well as on tRNA synthesis in mammary tissue. (Studies on mammary tRNA are a collaboration with Drs. A. Peacock and M. Green, LMC, NCI).

Significance to Biomedical Research and the Program of the Institute: Major unresolved questions in biology are whether tRNA may play a role in cellular regulation and in carcinogenesis. Approaches to understanding these problems are to determine if specific isoacceptor aa-tRNAs can influence the rate of protein synthesis and if misreading of isoacceptor aa-tRNAs in protein synthesis can induce cell transformation.

Proposed Course: The proposed course is: 1) to continue investigating the function of the minor Seryl-tRNAs and their possible role in lactation and 2) to perfect techniques for resolving isoacceptors from mammary tissue to determine the effects of hormone treatment on tRNA expression.

Publications:

Hatfield, D., Richer, L., Lyon, J. and Rice, M.: AA-tRNA populations in mammalian cells. Part II. Relative utilization of mammalian Lys-tRNA isoacceptors in protein synthesis. FEBS Letters 113: 249-252, 1980.

Hatfield, D., Rice, M., and Mushinski, J. F.: Comparison of the codon recognition properties and of the utilization of normal and tumor specific Phe-tRNAs in protein synthesis. Cancer Letters 12: 251-258, 1981.

Wilson, M., Hatfield, D. and Poirier, L.: Aminoacylation of ethionine to rat liver $tRNA^{Met}$ and its incorporation into protein. FEBS Letters 128: 157-160, 1981.

Diamond, A., Ducock, B. and Hatfield, D.: Structure and properties of a bovine liver UGA suppressor serine tRNA with a tryptophan anticodon. Cell 1981 (In Press)

Smith, D.W.E., McNamara, A., Rice, M. and Hatfield, D.: The effects of a post-transcriptional moditication on the function of $tRNA^{Lys}$ isoaccepting species in translation. J. Bio. Chem. 1981 (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04525-09 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on Electrophoretic Techniques for Protein, RNA, and DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. C. Peacock Chief, Protein Section LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new method for estimation of chain lengths of ribonucleic acids is under development. The method involves partial heat degradation of the RNA under study and measurements of the remaining and degraded molecules. From similar measurements on reference RNAs the chain lengths may be deduced. In addition, computer simulation permits computation of the relation between chain length and electrophoretic mobility for use in electrophoretic analysis of polydisperse samples. Heterogeneous nuclear RNA from HeLa cells has been studied by these techniques. The number-average RNA chain is approximately 5,000 nucleotides long; the weight-average is about twice as great.

Project Description

Objectives: To investigate the influence of primary structure and conformation on the electrophoretic and biological properties of proteins and nucleic acids and to use this information in devising improved methodology for analysis and purification. To study the role of hnRNA in cellular control in normal and malignant cells by these techniques. To develop suitable methodology for the preparation of hnRNA in undegraded and native form.

Methods Employed: Cultures of HeLa cells, assay of radioisotopes, isolation and electrophoresis of RNA and DNA, optical scanning of stained and unstained gels, preparative electrophoresis, optical measurement of melting of nucleic acids, ultracentrifugal analysis of RNA and DNA.

Major Findings: It was found that conformational differences between large and small RNA molecules were of sufficient importance to require revision of standard electrophoretic methods. The most important finding was that at electrophoretic field strengths of approximately 10 volts per centimeter, the largest RNA molecules migrated with the same mobility, and were not separated according to their size. In addition, composite gels of agarose and acrylamide resolved some test molecules which differed only in conformation, an undesirable property. These problems were overcome by the use of low concentrations of agarose as the gel medium, and by the use of much lower voltage gradients and an extended period of migration.

These changes in analytical technique, coupled with improved methods of computer analysis, have permitted tentative estimates of the size of the hnRNA transcribed in HeLa cells. The number-average RNA chain is 5500 nucleotides; the weight-average number is 12,000. Thus, though there are many chains of relatively short length, most of the mass of RNA is contained in longer chains. About 20% of the chains are 20,000 nucleotides long, or longer. The maximum size observed was 50,000, but there is substantial experimental uncertainty about the largest, least frequent species.

These size parameters were essentially constant over a labeling period of .5-3 hours, although steady state labeling had not been achieved. This result, requiring more analysis, indicates that the shaping of the RNA population is achieved rapidly.

The hnRNA population, as a whole, migrates more rapidly for its size than do members of the ribosomal RNA series. This finding indicates that there are conformational differences that persist in our analytical electrophoresis. Although there is at present no way to eliminate this condition, it is now possible to use reference materials to calibrate the gels and to use the method for the sizing of heterogeneous populations of hnRNA.

Significance to Biomedical Research and the Program of the Institute: The ability to estimate chain lengths of polydisperse ribonucleic acids by methods dependent on molecular properties very different from those previously available (centrifuge, electron microscope, etc.) provides a check on these methods. The improved electrophoretic separation of these large molecules presents them in a form suitable for further characterization by defined probes. Such methods should lead to improvements in understanding the control of gene expression in normal and malignant cells.

The heterogeneous RNA is the primary transcription of the gene and, in addition to specific messengers for specific proteins, presumably contains all RNA that might be involved in the control of genetic expression. Analytical methods for studying this RNA and assessing its role may be fundamental to understanding the alterations which occur in malignancy.

Proposed Course: 1) Efforts to develop a conformation-insensitive method for the electrophoretic analysis will continue. Efforts will be directed towards methods which do not accelerate degradation. 2) Properties other than size which may characterize the RNA population will be studied. These include self-association and ability to form duplexes with other molecules.

Publications:

Hooper, D. C., Steer, C. J., Dinarello, C. A., and Peacock, A. C.: Haptoglobin and albumin synthesis in isolated rat hepatocytes. Response to potential mediators of the acute-phase reaction. Biochim. Biophys. Acta. 653: 118-129, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04554-07-LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Development of In Vitro System for Studying Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI
OTHER:	T. Hirakawa	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop a reliable, rapid, convenient, and quantitative system for assay of malignant transformation and mutation in the cells of different species and origins in order to provide the optimum system for studying the mechanism of cell transformation as well as for obtaining the assay system for assessment of human risk against environmental carcinogens. This project may also lead to finding the crucial factors involved in the cell transformation. The skin fibroblasts derived from the patients with familial polyposis and Bloom's syndrome were examined for their behavior in culture to find no unique features compared to the normal skin fibroblasts. Further studies are in progress to determine whether the cells from these patients who are genetically predisposed to higher incidence of cancer are highly sensitive to the transformation as well as mutation. A protocol for quantitative and efficient system for assay of dominant mutation of human diploid fibroblasts was established using diphtheria-toxin resistance marker after extensive examination of experimental conditions. The factors contained in the serum which markedly affect the frequencies of chemically induced transformation are being investigated.

Project Description

Objectives: To establish an optimum system for quantitative assay of chemically induced transformation and mutation of human and mouse cells. Current interests are (1) to determine whether the skin fibroblasts derived from the patients with familial polyposis or Bloom's syndrome who are genetically predisposed to higher incidence of various type of cancer are highly susceptible to the chemically induced transformation, (2) to establish an efficient and convenient assay system for dominant mutation in human diploid fibroblasts, and (3) to identify the factors contained in the serum which markedly affect the frequency of the chemically induced transformation.

Methods Employed: Standard tissue culture techniques of our laboratory. Transformation assays previously developed by us (Int. J. Cancer 12, 463, 1973 and Proc. Natl. Acad. Sci., U.S.A. 75, 1334, 1978). Mutation assays measuring conversion of diphtheria toxin or ouabain-sensitive cells to resistant cells.

Major Findings: 1) The frequency of malignant transformation by carcinogens in the currently available system is so low that quantitation of transformation is very difficult. In order to obtain human cells sensitive to the induced transformation, human cell strains which were independently derived from the skin biopsy from patients with familial polyposis and Bloom's syndrome who are genetically predisposed to higher incidence of cancer were carefully examined as to their behavior in culture. Their growth pattern (cell doubling time, saturation density, and serum requirement) and growth morphology varied among strains, but none of them were beyond the level of variations observed with normal human skin fibroblast strains. These observations are partly contradictory to the report by others that the fibroblasts from familial polyposis patients showed higher saturation density and random cell arrangement in culture. Further studies are planned to examine these from patients with familal polyposis cells for their susceptibility to spontaneous and induced transformation and mutation.

2) A quantitative system for assaying of induction of mutation of human diploid fibroblasts was developed using diphtheria toxin-resistance as a marker. The optimum conditions were determined for the concentration of diphtheria toxin to select mutants, the expression time after carcinogen treatment, the cell densities at treatment and at selection, and the necessity of replating the treated cells before selection. The high cell density at the selection caused marked reduction of the recovery of the mutant, although metabolic cooperation was theoretically not expected to occur between mutant and unmutated cells because diphtheria toxin resistance mutation is dominantly expressed. A protocol of a quantitative system for assay of diphtheria toxin resistance mutation in diploid human fibroblasts has been established. On the other hand, untreated mouse Balb/3T3 lines (and their subclones) showed very high resistance to diphtheria toxin and required such a high concentration of the toxin to select the mutant that it was impractical to use this toxin for mutation assay in Balb/3T3 cells.

3) The variation of quality of commercially available serum is so large among the different batches of products that the transformation frequency varies significantly with each batch of serum used. The inconsistency and unpredictability of the serum quality has been one of the major obstacles in estab-

lishing a transformation assay system which gives reproducible and constant results, because all the target cells used for transformation require serum in culture medium for their growth. In order to determine the critical factors in the serum, several different batches of fetal calf serum were examined for their effects on the frequency of chemically induced transformation of A31-I-1 cells and then divided into two groups according to their effects, i.e., supportive or nonsupportive for induction of transformation. The crucial factors in serum, which are supposed to be different between two serum groups, are now under investigation.

Significance to Biomedical Research and the Program of the Institute: This project will provide a system for studying the mechanism of the cell, transformation and mutation and their relationship. It would also provide information on differences in the response to the carcinogens between different species and between different cell systems such as freshly isolated cells vs. established-line and *in vitro* vs. *in vivo*. The information thus obtained would be very helpful for extrapolating the results of animal experiments or *in vitro* systems into assessment of human risk against environmental carcinogens.

Proposed Course: 1) To continue studies to determine whether the cells derived from the patients who are genetically predisposed to higher incidence of cancer are highly sensitive to the transformation in culture. If they are more sensitive than normal cells, attempts will be made to develop a quantitative system for assay of transformation by chemical carcinogens using these cells and to find the crucial cause for their difference from normal cells. 2) To continue to identify the factors contained in serum which affect transformation frequency by pursuing the difference between the transformation-supportive and nonsupportive serum. 3) To pursue the goals outlined in Objectives and to publish results obtained.

Publications:

Bennedict, W.F., Chouroulinkov, I., Fisher, P.B., Kakunaga, T., Marquardt, H., Pienta, R.J. and Yamasaki, H.: Transformation in cell culture. In Montesano, R., Bartsch, H. and Tomatis, L. (Eds.): *Long-term and short-term screening assays for carcinogens: A Critical Review*. (IARC Monographs Suppl. 2). IARC, Lyon, 1980, pp. 185-199.

Kakunaga, T., *In vitro* chemical carcinogenesis of human cells. *Metabolism*, 17: 1323-1335, 1980.

Kakunaga, T.: Approaches towards the development of human transformation assay system. In Mishra, N.K., Dunkel, V. and Mehlman, M.A. (Eds.): *Advances in Environmental Toxicology; Mammalian Cell Transformation by Chemical Carcinogens.* Senate Press, Princeton Junction, pp. 355-382, 1981.

Kakunaga, T.: Assay of chemically-induced transformation of human cells. In Stich, J.F. and San, R.H.C. (Eds.): *Short Term Tests for Chemical Carcinogens.* New York, Springer-Verlag. In press.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04555-07-LMC

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Factors Affecting Malignant Transformation and Mutation by Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI
OTHER:	T. Hirakawa	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to clarify the mechanism of cell transformation by examining the genetic and physiological factors which affect cell transformation induced by chemicals and irradiation. Characterization of cell variants of Balb/3T3 line showing different susceptibility to the chemically or ultraviolet (UV) induced transformation, which have been isolated in our laboratory indicate that variants are different in the expression process by which promoting agents act. These results provide the first genetic evidence for the important role of a promoter-responsive step in the induction of cellular transformation. These variants will be very useful for studying the mechanism of the promotion process. On the other hand, a naturally occurring promoter, dihydroteleocidin B (DHTB), was found to be 100-fold stronger in its ability to enhance the carcinogen-induced transformation than TPA. The extraordinary enhancement of transformation by DHTB is ascribed to its resistance to metabolic inactivation which results in continuous exposure of the cells to active promoter. This suggests that many biochemical changes induced by a single application of promoter may not be directly involved in the promoting process.

Project Description

Objectives: To determine the process of malignant cell transformation induced by chemical and physical carcinogens by defining and identifying the genetic and physiological factors affecting the transformation.

Current interests are: (1) to clarify the cause of different susceptibility of cell variants to the chemical- and UV-induced cell transformation, (2) to determine the molecular mechanisms of promoters' action which are directly involved in the promotion of carcinogenesis.

Methods Employed: We used a quantitative system for assay of ouabain-resistance mutation (dominant) and malignant transformation (developed in our laboratory -- see project No. Z01-CP-04554) induced by UV in subclones of A31-1 cells derived from a Balb/3T3 mouse cell line. Competitive binding assay for determination of the number and binding kinetics of TPA receptors on the cell surface using $[^3H]$ phorbol dibutyrate. Assay of glucose transport across cell membrane using $[^{14}C]$ 2-D-deoxyglucose. Measurement of cell-to-cell comunication by examining transfer of action potential from cell to adjacent cell.

Major Findings: (1) Cell variants showing different susceptibility to the chemical- or UV-induced cell transformation, previously isolated in our laboratory, were further characterized. a) They showed similar susceptibility to the transformation by Kirsten murine sarcoma virus and the ouabain-resistance mutation by chemical carcinogens. b) Exposure of the carcinogen-treated resistant variant cells to promoting agents such as teleocidin and 12-O-tetradecanoyl phorbol-13-acetate (TPA) produced transformation at high frequency, whereas carcinogen treatment alone did not induce any transformation in this resistant line. The transformation frequency of the resistant cells treated with carcinogen plus promoter reached the level of the very sensitive cells treated with carcinogen. The promoting effect was strongest when the promoting agents were added to resistant cell cultures one week after the carcinogen treatment and was not observed when they were added prior to the carcinogen treatment. c) No differences were observed between variant cells in their abilities to metabolically inactivate TPA, the number and binding ability of TPA receptor on cell membrane, and the stimulation of glucose uptake and the decrease in the cell-to-cell communication in response to TPA-treatment. These results suggest that the fixation of transformation occurs at a similar high frequency in all the variants and that variants are different in the expression process of transformation by which promoting agents act.

(2) Dihydroteleocidin B (DHTB) was approximately 100-fold stronger than TPA in enhancing the transformation of A31-1-1 cells pretreated with 3-methylcholanthrene. rene. The enhancement of transformation was linearly proportional to the DHTB concentration and dependent on the duration of the DHTB exposure. On the other hand, there was no marked difference in the ability to stimulate DNA synthesis in G_0-arrested cells between DHTB and TPA. Metabolic inactivation of DHTB by A31-1-1 cells was markedly slower than that of TPA. The rapidity of metabolic inactivation was inversely proportional to the transformation enhancing potential between DHTB, TPA and phorbol-12,13-didecanoate. Uninterrupted presence of promoting agents was necessary for the enhancement of transformation,

whereas the repeated application of promoting agents failed to stimulate DNA synthesis and glucose uptake beyond the level induced by a single application of the agent due to the down regulation mechanism as have been reported. These results suggest that the acute effects of promoting agents which are sensitive to the down regulation, seem unrelated to their activity to enhance the transformation.

(3) The cell strains derived from skin biopsy taken from patients with familial polyposis or Bloom's syndrome who are genetically predisposed to a higher incidence of cancer were examined for their properties in culture to find the genetic factors involved in the cell transformation (see project No. Z01-CP-04554-07-LMC).

(4) The factors contained in serum which affect the frequency of chemically induced transformation of Balb/3T3 cells were investigated (see project No. Z01-CP-04554-07-LMC).

Significance to Biomedical Research and the Program of the Institute: This project will provide (1) information on the development and improvement of the system for assay of chemical and physical carcinogens and mutagens; (2) information about the mechanism of cell transformation by carcinogens; and (3) basic information about the environmental, genetic, and physiological factors in the incidence of human cancer. Thus, this project directly aims at the aspect of the Program of the Institute which seeks to find rapid meaningful assays for environmental carcinogens and ways to prevent cancer incidences.

Proposed Course: (1) To continue to investigate the molecular basis for genetic difference in the susceptibility of A31-1-1 cell variants to chemically induced transformation with special emphasis on the process by which promoting agents act. (2) To identify the biochemical effects of promoters which are directly involved in the promotion effects of promoting agents with emphasis on the mechanism of the requirement of continuous exposure to active promoters for enhancing the transformation which can not be explained by the already known effects of promoting agents. (3) To pursue the goals outlined in Objectives and to publish the results obtained.

Publications:

Kakunaga, T. and Crow, J.D.: Cell variants of different sensitivity to ultraviolet light-induced cell transformation. Science, 209: 505-507, 1980.

Kakunaga, T., Lo, K.Y., Leavitt, J. and Ikenaga, M.: Relationship between transformation and mutation in mammalian cells. In Gelboin H.V., Ts'o, P.O.P., and Pullman, B. (Eds.): Carcinogenesis: Fundamental Mechanisms and Environmental Effects. Reidel, Dordecht, Holland, 1980, pp. 527-541.

Kakunaga, T.: Cell transformation by chemical agents. In Yamane, I., Okada, Y., and Kuroki, T. (Eds.): Somatic Cell Genetics. Rikogakusya Publishing, Tokyo. (In Press)

Kakunaga, T.: Mechanisms of cell transformation by chemical carcinogens - Relation to the mutation. Tissue Culture. (In Press)

Kakunaga, T.: Mammalian cell mutagenesis and carcinogenesis *in vitro* by chemical carcinogenesis. In Inve, N. and Kuroki, T. (Eds.): The Use of Mammmalian Cells for Detection of Environmental Carcinogens, Mechanisms and Application. Baltimore, University Park Press. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04578-05 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Genes and Their Products Responsible for the Transformed Phenotype

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI
OTHER:	H. Hamada	Visiting Associate	LMC	NCI
	T. Kirakawa	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)
J. Leavitt, Division of Virology, FDA
K. Weber, Max Planck Institute, Goettingen, West Germany

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.3	PROFESSIONAL: 1.4	OTHER: 0.9

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long-range goal of this work to clarify the nature of cellular molecules responsible for the malignant phenotype of chemically transformed cells by using new techniques in molecular biology. When the protein molecules were compared by two-dimensional gel electrophoresis between the chemically transformed human fibroblasts and normal parent cell, one new polypeptide was recognized in the transformed cells. This novel polypeptide was identified as a product of mutated B-actin gene by immunoprecipitation with anti-actin antibody, comparison of its tryptic peptide patterns, isolation of mRNA coding for the new protein, cloning of DNA complementary to actin mRNA from the transformed cells, determination of size of actin mRNAs, complete amino acid sequencing of normal and altered B-actin molecules, and Southern blotting analysis of actin gene in the transformed cells using a recombinant DNA containing actin cDNA. These results provide the first molecular evidence for an occurrence of mutation in the chemically transformed cells. Some results were obtained which indicate the close correlation of production of mutated B-actin with the expression of the transformed phenotypes.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To learn about genetic regulation of the expression of transformed phenotype and to determine the biochemical nature of the macromolecules controlling the expression of the transformed phenotype. Current interest are: 1) to understand the molecular mechanism of the polypeptide changes associated with the transofrmation of human cells; 2) to determine the relationship between the particular polypeptide changes and the expression of the transformed phenotype in the transformed cells; 3) to establish a system for transfection in human diploid fibroblasts using biological and biochemical markers, and 4) to determine whether the transformed phenotype is expressed in a complete hybrid cell between a normal human diploid fibroblast cell and its chemical-transformat, and to determine whether DNA extracted from chemically transformed human cells will transfer the malignant phenotype into normal diploid human cells.

Methods Employed: Standard tissue culture techniques of our laboratory, karyotype analysis using chromosome banding techniques, transplantation in the cultured cells into nude mice, DNA transfection, and biochemical techniques such as two-dimensional gel electrophoresis, *in vitro* translation, amino acid sequencing, ultracentrifugation, DNA-DNA and DNA-RNA hybridization, and other techniques used for gene cloning and recombinant DNA work. Hybrid cells are isolated by selecting and cloning the growing cells in the presence of HAT (hypoxanthin, aminopterine, thymidine) and ouabain after fusing normal human diploid cells (ouabain-sensitive and HAT-resistant) with chemically transformed human cells which are ouabain-resistant and HAT-sensitive.

Major Findings: 1) When mRNA was prepared from the chemically transformed human fibroblasts and normal parent cells and translated into protein in *in vitro* translation system, and then the translation products were analyzed by two-dimensional gel electrophoresis, mRNA species which codes for a novel actin protein was identified in HuT-14 cell line, one of the 4NQO-transformed human fibroblast cell lines, but not in other cells.

2) pcHa-1, a recombinant plasmid containg human actin cDNA was constructed by ligating pBR322 DNA with the DNA complementary to mRNA which codes for human actin in HuT-14 cells. The size of the actin cDNA insert in pcHa-1 was estimated about 1,400 bp from its electrophoretic mobility on agarose gel.

3) pcDd actin ITL-I DNA, a recombinant plasmid DNA which contains a DNA sequence complementary to actin mRNA of Dictiostelium discodeum, hybridizes with both the altered actin mRNA and the β-actin mRNA but not the -actin mRNA. Hybridized mRNA was identified by analyzing *in vitro* translation products in the presence of the mRNA using two-dimensional gel electrophoresis. In contrast, pcHa-1 DNA hybridized to all three mRNA species. These results indicate (a) pcDd actin DNA sequence is similar to the human β-actin gene sequence and dissimilar to the human γ-actin gene sequence, (b) altered actin gene has sequences homologous with β-actin gene, and (c) cDNA sequence of pcHa-1 is a copy of altered or normal β-actin mRNA.

4) The approximate size of the β-actin mRNA was estimated to be about 2,200 nucleotides, and no difference was observed between normal and altered actin mRNA when their molecular size was determined either by sucrose gradient sedimentation or by methyl-mercury agarose gel electrophoresis. Because human β- and γ-actin polypeptides consist of 374 amino acids (see below), which correspond to 1,122 nucleotides of translated mRNA sequence, the above results indicate that about half of the actin mRNA consists of untranslated sequences.

5) Complete amino acid sequences of human normal and altered β-actin was determined. Normal and altered β-actin were isolated from normal and HuT-14 cells and purified using affinity chromatography and subsequently SDS-polyacrylamide gel electrophoresis. Altered β-actin differed from normal -actin by only a single amino acid substitution at position 244 (glycine to aspartic acid). This amino acid substitution corresponds to a point mutation GC-->AT transition.

6) Southern blotting of radioactive pcDd actin DNA to restriction endonuclease digested NuT-14 DNA produced only a single hybrid band (a 6 kb fragment with EcoR1 digestion and 12 kb fragment with BamH1 digestion), while the pcHa-1 DNA probe detected only one additional band (a 3 kb fragment with EcoR1 digestion). These results suggest that HuT-14 cells contain only one copy per haploid genome for altered or β-actin. All the results (1 to 5), when considered together, indicate that altered actinis the product of a mutated β-actin gene.

7) The highly malignant subclone, termed HuT-14-T, and the less malignant subclone, termed HuT-14-N, were isolated from UV-irradiated HuT-14 cells. HuT-14-T produced tumors in all nude mice within 2 weeks after injection of cells at the dose of 2 x 10^5 cells per animal, while parent HuT-14-N produced tumors in half of the animals after a long latent period and HuT-14-N failed to produce tumors at the dose of 2 x 10^6 cells per animal. The ratio of the mutated β-actin and the normal β-actin synthesized was much greater in HuT-14-T cells and less in HuT-14-N cells compared to the parent HuT-14 cells. This finding suggests an association between the production of mutated β-actin and the expression of the transformed phenotype.

8) The frequency of biochemical transformation of normal human diploid cells using ouabain-resistance as a marker was extremely low when the DNA extracted from ouabain-resistant human cells were transfected to the cells by the calcium phosphate precipitation method. In contrast, the frequency of biochemical transformation of mouse L cells under the same condition was high. Various modifications of the transfection methods, such as a post-treatment with dimethylsulfoxide, the use of synchronized cultures at various stages of cell cycle as a target cell, and co-transfection with plasmid DNA which contained the original locus DNA sequence of SV40, enhanced the frequency of ouabain-resistance transformation of L cells, but not of human diploid fibroblasts. The optimum conditions for transfection using the method of mechanical microinjection into cellular nuclei are being examined.

9) The hybrid cells were obtained by fusing the 4-nitroquinoline-1-oxide (4NQO)-transformed human fibroblasts with the normal parent cells and were confirmed to be 1:1 cell hybrid by examining chromosome banding patterns. All hybrid cells did not produce any tumors when injected into nude mice, whereas the transformed parent cells produced tumors at high frequencies. These results indicate that the expression of tumorgenic property is recessive in the hybrid cells.

Significance to Biomedical Research and the Program of the Institute: The results from this project will provide information on the structure and function of the genes and their products controlling the expression of the transformed state and will also contribute to the understanding of the mechanism of carcinogenesis. Thus, this project is directly aimed at that aspect of the program of the Institute that seeks to find an essential difference in the biochemical nature between normal and malignant cells and a possible way to prevent and cure cancer.

Proposed Course: 1) To detect, isolate, and characterize the cellular genes and their products controlling the expression of transformed or normal phenotype of human cells by using transfection and gene cloning techniques; 2) to determine the structure of the genes related to the synthesis of human actin and the determine the regulatory mechanism of its gene expression and to determine the role of actin in the maintenance of transformed or normal phenotype of human cells; 3) to pursue the goals outlined in Objectives and to publish the results obtained.

Publications:

Barrett, J.C., Sheela, S., Ohki, K. and Kakunaga, T.: Reexamination of the role of plasminogen activator in anchorage-dependent growth of chemically transformed hamster cells. *Cancer Res*. 40: 1438-1442, 1980.

Leavitt, J. and Kakunaga, T.: Expression of a variant form of actin and additional polypeptide changes following chemically induced *in vitro* neoplastic transformation of human fibroblasts. *J. Biol. Chem*. 255: 1650-1661, 1980.

Vandekerckhove, J., Leavitt, J., Kakunaga, T. and Wever, K.: Co-expression of a mutant actin and two normal and cytoplasmic actins in a stably transformed human fibroblast line. *Cell* 22: 893-899, 1980.

Hamada, H., Leavitt, J. and Kakunagao, T.: Mutated -actin gene: Co-expression with an unmutated allele in the chemically transformed human fibroblast line. *Proc. Natl. Acad. Sci., U.S.A.* (In Press)

Leavitt, J., Bushar, G., Mohanty, R., Kakunaga, T. and Ennis, F.A.: Interaction of the structural polypeptides of influenza virus with the cellular cytoskeleton during productive infection of human fibroblasts. In Bishop, D. and Compans, R. (Eds.): *Negative Strand Viruses*. North Holland/New York, Elsevier. (In Press)

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04782-11 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hormones and Breast Tissue Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. R. Green	Research Chemist	LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogensis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to contribute to the understanding of hormone action on metabolic processes in normal breast tissue under various conditions and to understand the failure of or aberrant response to hormones by hyperplastic or neoplastic breast tissue under the same conditions. Among the topics studied are the response of mammary tissue in culture to hormones in terms of nucleic acid, protein, phosphoprotein, sialoglycoprotein, made by mammary tissue in various stages of differentiation during preneoplasia and by glycosaminoglycan synthesis. The kinds of proteins and other macromolecules made by mammary tissue in various stages of differentiation during preneoplasia and by neoplastic mammary tissue are being analyzed. Analyses of rodent and human milks, explants and formal in-fixed rodent and human breast tissue are in progress. Of particular interest are the post-translational modifications of proteins which occur during differentation and neoplasia.

Project Description

Objectives: To gain a clearer understanding of the interaction of hormones with breast tissue. The topics of interest are interaction of hormones such as insulin, hydrocortisone and prolactin with breast tissue, and the metabolic effects of these interactions. Responses by breast tissue in culture to prolactin, carcinogens and steroids in terms of nucleic acid synthesis and synthesis of macromolecules such as phosphoproteins, sialoglycoproteins, glycosaminoglycans and proteins are under investigation, as is modulation of these responses in mouse mammary tumor virus containing tissues and in neoplastic tissue. Rodent tissues are examined for preneoplastic changes following carcinogen treatment.

Methods Employed: Culture of mammary tissue explants in vitro using defined medium, isolation and characterization of nucleic acids, separation on agarose-acrylamide gels and on gradients, radioactive tracer techniques, isolation and purification of proteins from milk and explants, measurement of enzymatic activities, polyacrylamide gel electrophoresis, histochemical analysis of gels and tissue sections, autoradiography, binding of hormones to macromolecules.

Major Findings: Two phosphorylated glycoproteins of apparent molecular weight 60,000 and 68,000, one in mouse colostrum, the other in human milk, have been identified using histochemical procedures and chemical analyses. The proteins differ in chemical characteristics and in their presence in milk throughout lactation. The 68,000 M.W. protein in human milk was present in all samples of individual milks obtained from mothers 2 days to 11 months post-partum. It was shown to be a phosphoprotein by its blue reaction with the metachromatic dye, Ethyl Stains-all (ESA) and the partial loss of this staining reaction following treatment with phosphatase. The isolated protein was found to contain 10 nmoles per nmol of protein.

The 60,000 M.W. protein in mouse colostrum behaved similarly to the human protein with respect to staining properties with Ethyl Stains-all. The protein was highly visible in mouse milks 1-2 days post-partum.

Both proteins contain carbohydrate as shown by their reactivity with periodic acid-schiff base (PAS). The blue-green ESA staining property, following acid phosphatase digestion of both proteins, indicated the presence of additional acidic substituents. In the case of the mouse protein, the ability to stain blue-green was lost following treatment of the preparation with neuraminidase.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the constituents of breast tissue and milk is essential for determining changes that occur during the neoplastic process, for identifying breast tissue cells in culture by means of fluorescent antibodies made to specific antigens, for developing radiommunoossays for recurrent breast disease, and for measuring production of mRNAs for particular proteins and their regulation by hormones. Human milk, composition has been studied over many years. The recognition of a novel phosphorylated glycoproteins by a simple staining method, illustrates the utility of this approach.

The many uses of the differential staining method have yet to be fully realized. Proteins can be recognized as phosphorylated or sialylated prior to purification and their purification can be monitored by means of this stain. Carcinomatous tissues may produce sialoglyco-proteins not made by normal tissues. These can be recognized in tissue sections and on gels following extraction of the tissue and electrophoresis of proteins.

Modification of proteins plays an important part in cellular interactions, hormone responsiveness, viral transformation and enzyme function. A variety of methods for demonstrating these modifying groups have been utilized. Among methods used for detecting phosphorus, isotopic labeling with P^{32} is commonly used. Glycoproteins containing sialic acid are also detected by labeling procedures and by the use of lectins. In situations such as analysis of tissues and fluids from humans, *in vivo* labeling may not be possible. In addition, proteins that are fully phosphorylated may not label, or may label *in vitro* by kinase reactions that do not occur *in vivo*. A method that detects phosphorus and/or sialic acid on proteins by use of a dye will be of value in numerous investigations.

Proposed Course: The function of the phosphorylated glycoproteins is not known at present and requires further study. Evidence from isotope incorporation experiments in mammary explants from mice indicate that a protein of molecular weight similar to the colostral protein is made *in vitro*. An antibody to the colostral protein that also immunoprecipitates the protein synthesized *in vitro* is required to establish this point. Similarly, an antibody to the human protein could answer questions of relatedness to the mouse protein and whether the protein is made by normal and tumor breast tissue. Interest in phosphorylated glycoproteins as a class has been stimulated by the finding that many of the lysosomal enzymes are phosphorylated glycoproteins and that heritable mutations in the genes for these proteins result in the mucopolysaccharidoses. The human protein, while present in milk in excess of that expected for an enzyme, might serve as a prototype for structural studies of this kind of protein.

Both hydrocortisone and prolactin are required for the full expression of milk protein production in mammary explants. Levels of control at the transcriptional and translational level will be examined. Protein systhesis and modification in response to hydrocortisone and/or prolactin in nuclear and cytoplasmic fractions will be assessed to determine which proteins, in addition to the major milk proteins, are altered.

Protein phosphorylation is altered following stimulation by hormones and during carcinogensis. An examination of mammary cell membranes could lead to an understanding of changes in enzymatic function and alteration of hormone responsiveness.

Publications:

None

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04785 11 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of DNA Repair Mechanisms in the Etiology of Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.S. Day, III	Research Physical Scientist	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study of defective repair of MNNG-(N-methyl-N'-nitro-N-nitrosoguanidine) produced damage was extended. One-fifth (17/88) of the human tumor cell strains tested comprises a group of cell strains (Mer$^-$) that differs from the remaining 71 tumor strains (Mer$^+$) or from normal human fibroblasts (also Mer$^+$) in the following ways: 1) inability to excise O^6-methylguanine from their DNA after being treated with MNNG; 2) relative inability to support the growth of MNNG-damaged adenovirus 5; and 3) sensitivity to being inactivated by MNNG or BCNU (Bis-chloroethyl-nitrosourea) in a reproductive way. The results may be relevant to uncovering the molecular reasons for success of certain bifunctional alkylating chemotherapeutic agents (BCNU, CCNU).

Project Description

Objectives: To learn more about DNA repair mechanisms in human cells and about their role in carcinogenesis. In particular, to determine the nature of the DNA repair defects both in human tumor cells and in cells from persons who are genetically predisposed to cancer. In addition, to use human cell strains with characterized defects to study the mechanisms of action of carcinogens or suspect carcinogens in altering DNA.

Methods Employed: Using an adenovirus-host cell reactivation assay developed previously in this project, it has been possible to quantitate the deleterious effects of various chemical and physical treatments on the ability of the virus to initiate and sustain infection. The method involves establishing monolayer cell cultures which are infected with treated or non-treated adenovirus. The infected cells are then incubated 12-20 days with feeding by means of periodic overlaying with a nutrient agar. Non-treated virions or those treated ones which have been "reactivated" by cellular repair mechanisms form countable plaques of dead, lysed cells. The plaquing efficiency of unirradiated adenovirus is approximately the same on all of the non-transformed human cell strains so far tested. It is 0.1 to 100-fold greater when human tumor lines of various types are used. To quantitate DNA base damage due to MNNG, methylated purines (released from DNA by combined acid, high temperature treatment of DNAs extracted from ^{14}C MNNG-treated cells) were separated by high pressure liquid chromatography.

Major Findings:

1) Seventeen of 88 cell strains prepared from human tumors were identified as less able to support the growth of MNNG-treated adenoviruses than the remaining 71. (For the sake of brevity of exposition, the phenotype so identified -- characteristic of the 17 strains -- is termed Mer$^-$. The phenotype shown by the other 71 is, therefore, called Mer$^+$.)

2) Seven of 11 strains of human fibroblasts transformed by SV40 also showed the Mer$^-$ phenotype, whereas 38 strains of non-transformed human fibroblast strains (including the parent strains of four of the Mer$^-$ SV40 transformed strains) were Mer$^+$.

3) Fibroblast cultures (of non-transformed appearance), prepared from two patients whose tumors gave rise to Mer$^-$ cell strains, were found to be Mer$^+$.

4) A HeLa cell variant and an SV40-transformed cell line, found by others to be highly mutagenized by alkylating agents, were found to be Mer$^-$, whereas another HeLa variant and another SV40-transformed cell line, more resistant to such mutagenesis, were Mer$^+$.

5) Fourteen strains having the Mer$^-$ phenotype were unable to remove O^6-methylguanine (produced in DNA by MNNG) from their DNA, whereas 8 Mer$^+$ strains were able to perform such repair.

6) In collaboration with Dr. Dominic Scudiero (now at FCRC), it has been determined that most cell strains having the Mer$^-$ phenotype are much more sensitive to being inactivated reproductively (colony forming ability) by MNNG than are Mer$^+$ cell strains. Similarly, Mer- strains are also sensitive to inactivation by BCNU, or methylmethanesulfonate.

7) MNNG-pretreatment of some but not all Mer$^+$ tumor cell strains reduced their ability to support the growth of MNNG-treated adenoviruses. The effect may be due to saturating cellular O^6-methylguanine repair capacity.

Significance to Biomedical Research and the Program of the Institute:

The results of this project suggest the possibility that a fraction, one-fifth of all human tumors, is composed of repair-defective cells. Certain bi-functional alkylating agents (BCNU, CCNU) are known to be extremely effective against some human tumors and to be relatively inneffective against others, also indicating the possibility that the molecular basis for the success of alkylation chemotherapy will soon be elucidated. A study of excision of O^6-methylguanine from MNNG-treated primary human tumor biopsies may provide evidence for the proof or disproof of this possibility. Physical, chemical, and viral carcinogens are all known to alter the structural integrity of the cellular genetic apparatus. An evaluation of the role of DNA repair and/or related mechanisms in conferring resistance or susceptibility to mutagenesis and carcinogenesis is an important facet in any overall program having as its goal the understanding of the molecular pathways which, when perturbed, give rise to carcinogenesis in humans. It is the long-range goal of this project to determine whether or not the elucidation of genetic repair mechanisms is important to the understanding of carcinogenesis. It is expected that an understanding of human repair mechanisms, in general, will benefit many areas of biomedical research.

Proposed Course: To pursue the goals outlined above in Objectives and to publish these results.

Publications:

Day, Rufus S., III and Ziolkowski, C.: An MNNG-produced cellular response resulting in decreased survival of MNNG-treated adenovirus 5. In Altmann, H., Riklis, E. and Slor, H. (Eds.): Proceedings of International Symposium on the IGEGM: DNA Repair and Late Effects. Tel Aviv and the Dead Sea, May 1978. Nuclear Research Center, Negev, 1980, pp. 93-102.

Day, Rufus S., III, Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S.A., Lubiniecki, A., Girardi, A., Galloway, S.M., and Bynum, G.D.: Defective repair of alkylated DNA by human tumor and SV40 transformed human cell strains. Nature 288: 724-727, 1980.

Day, Rufus S., III, Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S.A., and Mattern, M.R.: Human tumor strains with abnormal repair of alkylation damage. In Gelboin, H.V., MacMahon, B., Matsushima, T., Sugimura, T., Takayama, S., and Takebe, H. (Eds.): Genetic and Environmental Factors in Experimental and Human Cancer Proceedings of the Tenth International Symposium of The Princess Takamatsu Cancer Research Fund, Tokyo, 1979 . Tokyo, Japan Scientific Societies Press, 1980, pp. 247-257.

Day, Rufus S., III: Studying DNA repair using human adenovirus 2 and 5: Purification, plaque assay, and inactivation. In Friedberg, E.C. and Hanawalt, P.C. (Eds.): DNA Repair: A Laboratory Manual of Research Procedures. Vol. 1, Part B. New York, Marcel Dekker, 1981, pp. 587-604.

Day, Rufus S., III: The photobiology of the DNA viruses lambda, T4, and øx174. In Graenckel-Conrat, H. and Wagner, R.R. (Eds.): Comprehensive Virology, Vol. 17. New York, Plenum, 1981, pp. 353-449.

Day, Rufus S., III and Ziolkowski, C.H.J. MNNG-pretreatment of a human kidney carcinoma cell strain decreases its ability to repair MNNG-treated adenovirus 5. Carcinogenesis 2: 213-218, 1981.

Day, Rufus S., III and Ziolkowski, C.H.J. UV-induced reversion of adenovirus 5ts2 infecting human cells. Photochemistry and Photobiology. In Press.

Slor, H., Mizusawa, H., Neihart, N., Kakefuda, T., Day, R.S., III, and Bustin, M.: Immunochemical visualization of the binding of a chemical carcinogen to the genome. Cancer Research. In Press.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05001-04 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of DNA Damage and its Repair Mechanisms in In Vitro Chemical Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.Y. Lo	Senior Staff Fellow	LMC	NCI
Other:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The repair of DNA damage induced by benzo(a)pyrene diol epoxide I (BPDEI, anti) were examined in the fibroblasts derived from human normal individual (KD) and xeroderma pigmentosum patients (XP). Cells were first treated with $[^3H]$BPDEI in Dubecco's phosphate buffered saline (PBS) at 4°C and were then post-incubated with BPDEI-free depleted medium at 37°C for various lengths of time. The removal rate of BPDEI-DNA adduct in both KD and XP cells showed a biphasic kinetics - a fast repair phase followed by a slow repair phase. The addition of exogenous pBR322 DNA to the cell lysates prepared from the cells within 1 hr. after $[^3H]$BPDEI treatment resulted in the transfer of radioactivity to the plasmid DNA. Since complete hydrolysis of BPDEI occurs within 7-8 min in DNA solutions at pH 7.0, 0°C, our findings suggest that some cellular components can stabilize BPDEI, which in turn interact with the exogenous DNA to form BPDEI-DNA adduct. The nature of such cellular components is currently under investigation.

Project Description

Objectives: To study the biochemical mechanism of *in vitro* transformation by chemical carcinogens with special attention to the role of DNA damage and its repair mechanisms in this process. Current interests are 1) to measure the excision repair of DNA damaged by benzo(a)pyrene diol epoxide in human KD and XP cells, and 2) to investigate the nature of cellular components which stabilize BPDE in cells.

Methods Employed: Cell culture, radioactive tracer, RIA, DNA isolation, ethidium bromide-cesium chloride centrifugation, agarose gel electrophoresis, Southern blot, column chromatography, and HPLC.

Major Findings: 1) The removal of covalently bound BPDEI-DNA adduct in both KD and XP cells showed biphasic kinetics (i.e., a fast repair phase followed by a slow repair phase). 2) The carcinogen-DNA adduct formed in both KD and XP cells was identified as BPDEI-deoxyguanosine by HPLC. 3) Exogenous pBR322 DNA was added to the lysis mixtures for the isolation of total DNA from [^{3}H] BPDEI-treated cells, and then the plasmid DNA and cellular DNA were purified and separated. Radioactivity was found in the plasmid DNA mixed with the lysates of cells which were harvested within 1 hr. after the carcinogen treatment, but was not found in the pBR322 DNA included in the lysates of cells which had been post-incubation with BPDE-free medium at 37°C for several hours. These results suggest that BPDEI, which hydrolyzes completely to tetrols within 7-8 min at pH7.0, 0°C is stabilized by cellular components in cells, and the stabilized BPDE can react with exogenous DNA as well as cellular DNA after the lysis of cells. 4) The nature of the cellular components which hold BPDE in stable condition is currently under investigation.

Significance to Biomedical Research and the Program of the Institute: The results from this project will provide information about the mechanism of cell transformation, in particular the process of initiation of transformation by chemical carcinogens.

Proposed Course: To pursue the goals as outlined in objectives.

Publications:

Lo, K. and Kakunaga, T.: Only one type of Benzo(a)pyrene-DNA adduct is Detected in transformable mouse cells. *Biochem. Biophys. Res. Commun.* 99: 820-829, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05068-03 LMC

PERIOD COVERED

October 1, 1980 to September 31, 1981

TITLE OF PROJECT (80 characters or less)

HnRNA in Normal and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. C. Peacock	Chief, Protein Section	LMC	NCI
OTHER:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: .2	OTHER: .8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Measurements of the amount of steady-state hnRNA and parameters characterizing its size distribution may be of value in understanding the biomedical properties of malignant cells. The populations of hnRNA found in several transformed and normal 3T3 cells were compared, using three different labeling times, using improved electropheretic and computational methods. The hnRNA populations from normal and malignant cells were very similar.

Project Description

Objectives: To characterize a variety of neoplastic and normal cells as to their content of hnRNA, both with respect to amount and size parameters. Normal cells subject to perturbation, as by hormones (e.g., mammary tissue, prolactin), will also be studied to learn whether hnRNA can play any role in cellular regulation.

Methods Employed: Cell culture, RNA, DNA analysis by electrophoresis, and RNA size distribution by thermal degradation (Project No. Z01-CP-04525-09-LMC).

Major Findings: During the course of this work, extensive improvements in the characterization of hnRNA were made and early exploratory studies on the hnRNA distribution in normal and malignant cells were redone. Because of variability in preparations of hnRNA from these cells, a statistical approach was taken. Detailed analysis of these results is in progress, but it is clear that any differences among these cell types is very small.

Significance to Biomedical Research and the Program of the Institute: A common assumption is that the altered behavior of malignant cells is a result of mutation of the DNA. It is also believable, however, that malignant behavior could be a result of control aberrations alone. This work will help to discriminate among these hypotheses.

Proposed Course: 1) Further detailed analysis of hnRNA of the cell types described above; 2) the effect of hormones and promoters will be investigated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05081-03 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of DNA Synthesis in Diploid and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. Mattern Senior Staff Fellow LMC NCI

COOPERATING UNITS (if any)

Division of Cancer Treatment, Laboratory of Molecular Pharmacology, NCI

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Experiments with adenovirus 5 indicated that the failure of malignant cells to discriminate against 5-bromodeoxyuridine as a DNA precursor has long-term biological significance. Several steps of DNA repair were examined through the effects of inhibitors of DNA polymerases and topoisomerases on the sedimentation of DNA nucleoids. DNA repair is blocked at its early stages (damage recognition and/or DNA relaxation) by inhibitors of type II DNA topoisomerases, and at its later steps (leading to regeneration of compact, supercoiled DNA) by inhibitors of both α and β DNA polymerases. Both topoisomerase inhibitors produce a 25-35% relaxation of nuclear DNA. Chinese hamster ovary cells that are partially resistant to novobiocin contain DNA nucleoids that sediment 20-30% more slowly that those from the novobiocin-sensitive cells. Their sedimentation is not affected by incubating the cells with novobiocin. The defect in tumor cells that cannot repair alkylation damage (Mer- cells) was found to be multifaceted in studies measuring the relaxation and recoiling of nucleoid DNA in conjunction with collaborative studies measuring induced and constitutive capacity to repair damaged viruses, repair DNA synthesis, and cytotoxicity.

Project Description

Objectives: To compare the mechanism and regulation of replicative and repair-type DNA synthesis in diploid mammalian cells and in transformed cells. Specific areas of study: 1) the relationship of higher-order DNA structure to DNA metabolism, and 2) alterations in DNA metabolism in malignant human cells and in specific classes of tumour and diploid cells, including the identification of altered proteins.

Methods Employed: DNA synthesis was assayed in intact cells by measuring the incorporation of radioactively-labeled thymidine (TdR) into TCA-insoluble material. 5-bromodeoxyuridine (BUDR) discrimination and excision of incorporated BUDR were determined according to published procedures. Excised BUDR moieties were identified and separated by high pressure liquid chromatography. Growth of adenovirus 5 in the presence of various ratios of TdR and BUDR and plaque assay of the recovered virus were carried out according to Day *et al*. Repair DNA synthesis was ascertained according to the BND-cellulose method of Scudiero *et al*. The survival of cells after UV, MNNG, or *m*-AMSA treatment was determined according to published procedures, as was the sedimentation of mucleoids in 15-30% neutral sucrose gradients. In repair experiments, cultures were treated with DNA polymerase or topoisomerase inhibitors for 60 min., exposed to a DNA-damaging agent, and incubated thereafter for various times in the presence of inhibitor to determine the kinetics of nucleoid relaxation and recoiling during repair. Proteins will be separated and identified by standard techniques of polyacrylamide gel electrophoresis.

Major Findings / Projected Studies:

1. Differences in DNA metabolism in diploid and transformed cells. Lack of discrimination against BUDR by transformed human cells. Adenovirus 5 was grown in both normal and malignant cells in the presence of varying relative amounts of TdR and its analogue, BUDR. The ability of the progeny virus to infect and produce viable colonies (plaques) in the normal and malignant cells was then determined. It was found that viruses produced originally in A549 cells (which do not discriminate against the incorporation of BUDR into their own nuclear DNA) in the presence of 2- to 20-fold excess BUDR: TdR grew less well when re-introduced into A549 cells than did virus produced under the same conditions in KD cells (which do discriminate) and even less well when introduced into KD cells. These data, along with those suggesting that in diploid cells, BUDR that is incorporated is later excised, indicateing that the phenomenon of BUDR discrimination, observed in nontransformed human cells upon incubation with exogenous DNA presursors, has physiological consequences and is not merely a reflection of prereplicative metabolism of the precursors. The question of excision of incorporated BUDR will be examined further by the use of alternate methods, including high pressure liquid chromatography and *in situ* hybridization of viral DNA (work with R. Day). A longer range goal of this work is to identify the factor(s) whose presence (or modification) in transformed cells results in their incapacity to discriminate against BUDR.

DNA metabolism in repair-deficient human cells. The several steps of DNA repair following treatment of cell cultures with biologically relevant amounts of UV light or alkylating agents such as MNNG were characterized by following changes in the *in vivo* supercoiling of DNA (sedimentation in neutral sucrose gradients); an initial DNA relaxation step was followed by changes in the DNA during the next 1-4 hours that produced nucleoids whose sedimentation rates approached, and, after 4-24 hrs., became equal to those of nucleoids from untreated control cultures. The latter reflects repair DNA synthesis, ligation, and probably the regeneration of supercoiled DNA. Incubation of the cultures with novobiocin or nalidixic acid for 1 hr. prior to UV or MNNG treatment and during subsequent repair prevented the initial relaxation step; when novobiocin was removed from contact with the cells, the DNA became relaxed. Treatment of damaged cells with cytosine arabinoside (α-polymerase inhibitor) or dideoxythymidine (β-polymerase inhibitor) permitted initial DNA relaxation but delayed restoration of supercoiled DNA for 1-4 hr. Neither inhibitor by itself produced a permanent repair block, but cells treated with both inhibitors could not complete repair even after 24 hrs. Thus, it is likely that 1) both and DNA polymerases are normally utilized at some stage of DNA repair, but 2) one type of polymerase may substitute for another, as in prokaryotic cells.

This method has been and is currently being used in the study of a class of tumor cells (20% of tumors) that are defective in the repair of alkylation damage (the "Mer$^-$" phenotype, R.Day). The defect in Mer$^-$ cells has been shown to be after DNA relaxation. Since the initial discovery of the repair defect through host cell reactivation studies with damaged adenovirus (Day *et al.*), it has become apparent that the defect is not a simple one, but has both quantitative and qualitative components. Experiments were carried out (with R. Day, NCI, and D. Scudiero, Frederick Cancer Research Center) to establish a correlation among 1) the delay or abolition of the later steps of DNA repair (as determined by the nucleoid sedimentation method), 2) induced or constitutive incapacity or diminished capacity to support the growth of virus treated with alkylating agents, 3) the amount of repair DNA synthesis per cell (which is generally greater in Mer$^-$ cells) and 4) cytotoxicity of alkylating agents. Such a correlation seems to hold, as demonstrated in experiments with several Mer$^-$ tumor lines. An additional repair-defective line has been found that, unlike the other Mer$^-$ cells, appears to lack the initial DNA relaxing capacity in response to MNNG treatment. This line has been characterized extensively with respect to its sensitivity to various inhibitors of DNA replication and repair. Several agents which bind to constituents of a hypothetical DNA repair complex (e.g., dideoxythymidine, novobiocin) induce DNA relaxation after MNNG treatment, suggesting that at least one of the molecular defects of this line may be in a component of the repair complex.

Attempts will be made to isolate such complexes (considered, as a first approximation, to be proteins associated specifically with damaged DNA) by banding in neutral sucrose gradients, with the objective of identifying the individual components and determining differences in the makeup of these complexes in tumor vs. normal, repair-proficient vs. repair-deficient tumor, and repair-proficient vs. repair-deficient non-tumor human cell lines.

2. Dependence of DNA metabolism upon higher order DNA structure. Initial demonstrations that novobiocin and nalidixic acid (which inhibit different subunits of bacterial DNA gyrase [topoisomerase II]) reversibly inhibit replicative DNA synthesis and, with even greater efficiency, repair-type DNA synthesis induced by agents that do not produce DNA strand breaks (UV light, MNNG) led to the hypothesis that the initiation of DNA replication and the initial step in the repair of some DNA lesions require a modification in DNA superstructure that is mediated by DNA topoisomerases (work with D. Scudiero). Additional evidence has been obtained in support of this model: (1) Concentrations of novobiocin and nalidixic acid that reversibly block DNA replication by 50% also reversibly relax DNA in nucleoids (supercoiled DNA structures produced by gentle lysis of cells at neutral pH), consistent with their inhibiting DNA supercoiling inside the cell. (2) Novobiocin and nalidixic acid block an early step in DNA repair (q.v. above); in the presence of these drugs, no DNA relaxation occurs after treatment of cells with UV light or MNNG. This result is consistent with the action of these drugs upon a topoisomerase rather than upon a DNA polymerase *in vivo* (both kinds of enzymes are blocked *in vitro* by novobiocin). (3) Chinese hamster ovary cells that were made partially resistant to novobiocin contain nucleoids that sediment at 70-80% of the rate of nucleoids from fully sensitive CHO cells in 15-30% neutral sucrose gradients. Thus, the novobiocin-sensitive target appears to affect the higher-order structure of DNA. This idea is supported further by the result that, unlike normal CHO cells, the ones resistant to novobiocin show no change in the sedimentation rate of their nucleoids upon incubation with novobiocin, although DNA replication inhibition experiments have demonstrated that the drug does enter the resistant cells.

It is proposed to extend these studies to include determining the effect of nalidixic acid upon DNA superstructure in the novobiocin-resistant CHO cells and to employ biochemical techniques for a) the separation and identification of proteins and b) purification of type II DNA topoisomerases which may be altered in the novobiocin-resistant cells (work with R. Day).

3. Response of human diploid and tumor cells to treatment with the DNA intercalating agent 4'(9-acridinyl-amino)-methanesulfon-m-amisidide (m-AMSA) (with L. Zwelling, K. Kohn, DCT, NCI). m-AMSA, a chemotherapeutic agent whose mechanism of action is largely unknown, has been shown in experiments with mouse leukaemic cells to produce single-strand breaks in the DNA; these breaks are associated with protein, and the protein could be a topoisomerase or other protein involved in the repair of damaged DNA. The mechanisms of formation and repair of AMSA-induced DNA damage in normal cells and whether there are differences in these mechanisms in transformed or repair-deficient human cells are being investigated.

Significance to Biomedical Research and the Program of the Institute: The way in which cellular DNA is replicated and maintained between duplications is altered in transformed cells. Understanding these alterations at the molecular and biological levels through comparative studies of DNA metabolism (replication and repair) will contribute to a greater understanding of the mechanism of malignant transformation as well as the "usual" mechanisms and regulation of these functions. The latter is of general biomedical interest.

Proposed Course: To continue the studies in the areas described and to communicate the results through appropriate professional vehicles.

Publications:

Day, R.S., III, Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S.A. and Mattern, M.R.: Human tumor strains with abnormal repair of alkylation damage. In Gelboin, H.V. et al. (Eds.): Genetic and Environmental Factors in Experimental and Human Cancer, Tokyo, Japan Sci. Soc. Press, 1980, pp. 247-257.

Mattern, M.R.: Differences between diploid and transformed cells in the incorporation of 5-bromodeoxyuridine into DNA. Exp. Cell Res. 128: 181-190, 1980.

Mattern, M.R. and Scudiero, D.A.: Dependence of mammalian DNA synthesis on DNA supercoiling III. Characterization of the inhibition of replicative and repair-type DNA synthesis by novobiocin and nalidixic acid. Biochim. Biophy. Acta 653: 248-258, 1981.

Mattern, M.R.: Differences between diploid and transformed human cells in the incorporation of thymidine and 5-bromodeoxyuridine into DNA. In Katz, R. and Cox, R .P. (Eds.): Intact Mammalian Cell Lines in Study of Inborn Metabolic Diseases Proceedings of the Workshop on Intact Mammalian Cell Lines in the Study of Inborn Metabolic Diseases, Bethesda, MD., 1980 . Washington, DC, U.S. Govt. Printing Office, 1981, (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05086-03 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less) Regulation of Benzo(a)pyrene Metabolism in Human Cells: Preparation and Identification of Monoclonal Antibodies to Carcinogen Metabolizing Enzymes of Human Placenta and Monocyte Microsomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sang Shin Park	Visiting Associate	LMC	NCI
OTHERS:	Harry V. Gelboin	Chief	LMC	NCI
	Tadahiko, Fujino	Expert	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.7	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Benzo(a)pyrene is metabolized either to nontoxic water soluble conjugates or to the ultimate carcinogen, BP-7, 8-diol-9, 10-epoxide. The direction of BP metabolism might be dependent on the presence of specific predominant forms of enzymes involved in the BP metabolism of individuals exposed to chemical carcinogens. Monoclonal antibodies (MAB) are specific to antigenic determinants. Therefore, Balb/c female mice were immunized with microsomes obtained from the placenta of women who smoke heavily and fractions of human blood monocytes induced with benzo(a)anthracene. The primed spleen cells were fused by polyethylene glycol with myeloma cells and grown in selective media to isolate hybridomas. 21/73 and 87/205 independent hybrid clones tested are positive for mouse immunoglobulin production to monocyte fractions and human placenta microsomes. The immunoglobulins produced by hybrids were types of IgG1 and IgM and inhibited aryl hydrocarbon hydroxylase activities of human placental microsomes by 15 - 45%. MAB reacting with human cytochrome P-450 would be very useful for the testing of cytochrome P-450 levels of individuals exposed to different environmental carcinogens.

Project Description

Objectives: Activation of benzo(a)pyrene is initiated by mixed function oxidases and further converted by epoxide hydratase to ultimate carcinogenic products as well as non-toxic water soluble conjugates. Several isozymes of cytochrome P-450 have been reported and a specific form appears to be predominant following induction by specific compounds. Some specific carcinogen metabolizing enzymes might be present as a predominant form in individuals who smoke heavily, as well as in those people exposed to carcinogenic environments. Microsomal preparations from the placentas of women who smoke heavily possess high levels of mixed function oxidases. Human monocytes induced with benzo(a)anthracene contained more than two times the mixed function oxidase activity of untreated monocytes. The purpose of this work is to prepare monoclonal antibodies to enzymes involved in BP metabolism to study the multiplicity of enzymes as well as to identify the predominant forms inducible by carcinogens.

Methods Employed: Balb/c female mice were immunized with human monocyte fractions and placenta microsomes from women who smoke heavily. The primed spleen cells and myeloma cells were fused by polyethylene glycol and the cells were seeded in wells containing selective HAT medium to obtain hybridomas producing monoclonal antibodies. The production of monoclonal antibodies was detected by solid-phase radioimmunoassay and the monoclonal antibodies were further characterized for the specifities of mixed function oxidases.

Major Findings: 1) Twenty-one out of 73 independent hybrid clones were producers of monoclonal antibodies to human monocyte fractions induced with benzo(a)anthracene. 2) Eighty-seven out of 205 independent hybrid clones were producers of monoclonal antibodies to human placental microsomes of women who smoke heavily. The immunoglobulins produced by hybrids were types of IgG1 and IgM and inhibited aryl hydrocarbon hydroxylase activities of human placenta microsomes by 15 - 45%. Some of the monoclonal antibodies also bind to 3-methylcholanthrene-induced rat cytochrome P-450.

Significance to Biomedical Research and the Program of the Institute: The multiple forms of the cytochrome P-450 are induced differentially and have an important role in benzo(a)pyrene detoxification and activation. Therefore, provided with a series of monoclonal antibodies to the enzymes involved in benzo(a)pyrene metabolism, not only will we have a new and powerful tool for chemical carcinogenesis but we will also be able to analyze the differences in individual susceptibility to cancer. In this way, we will have new tools for screening and preventing the occurrence of human cancer among populations exposed to environmental carcinogens.

Proposed Course: 1) Monoclonal antibodies to mixed function oxidase will be labeled with ^{125}I and fluorescein to develop radioimmunoassay and immunofluorescence techniques in order to determine the levels of enzymes involved in BP metabolism for individuals prone to chemical carcinogenesis. 2) Since the balance of BP metabolism is not only controlled by the cytochrome P-450 but is also determined by the enzymes epoxide hydratase, P-450 cytochrome reductase, glutathione S-transferase, glucuronic acid transferase, sulfotransferase and β-glucuronidase, monoclonal antibodies to those enzymes should be also developed.

Publications:

Park, S.S., Persson, A.V., Coon, M.J., Gelboin, H.V.: Monoclonal antibodies to rabbit liver cytochrome P-450 LM2. FEBS LETTERS 116: 231-235, 1980.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05089-03 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Effect of Human Liver Epoxide Hydrolase in DNA Binding and Benzo(a)pyrene Metabolism by P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. M. Gozukara	Visiting Scientist	LMC	NCI
OTHER:	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)
F. P. Guengerich, Associate Professor of Biochemistry,
Vanderbilt University of Tennessee

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of human liver epoxide hydrolase were examined by using ^{3}H(-)t-7, 8-diol BP as a substrate to generate diol epoxide I and II in the incubation medium, with uninduced and BA-preinduced human monocytes, lymphocytes, Fischer rat liver (TRL-2) cells, and Buffalo rat liver (BRL) cells. The binding of reactive metabolites to added and intact cellular DNA was determined. The epoxide hydrolase was added either to incubation mixtures or to tissue culture media and inhibition of DNA binding was determined.

In the second part of the project, major forms of cytochrome P-450 were purified from 3-methylcholanthrene (3MC), phenobarbital (PB), and β-Naphthoflavone (BNF) treated rat liver microsomes. A purified, reconstituted microsomal mixed function oxidase (MFO) system, containing different forms of cytochrome P-450, NADPH cytochrome c (P-450), reductase and dilauroylglycoryl 3-phosphocholine was used to analyze benzo(a)pyrene metabolism.

Project Description

Objectives: To determine the metabolism of ^{3}H(-)t-7, 8-diol BP and covalent binding of reactive metabolites to added and intact cellular DNA in tissue culture media. To demonstrate the unique positional specificities of BP metabolism by different forms of cytochrome P-450 which may regulate the balance between activation and detoxification pathways of polycyclic aromatic hydrocarbons (PAH).

Methods Employed: Fluorospectrophotometry, high pressure liquid chromatography, tissue culture techniques, slab gel electrophoresis and autoradiography.

Major Findings: Human monocytes, lymphocytes, Fischer rat liver (TRL-2) cells, and Buffalo rat liver (BRL) cells catalyze binding of ^{3}H(-)t-7, 8-diol BP to added and intact cellular DNA in tissue culture media. Cells that are preinduced by benzanthracene (BA) exhibit greater levels of DNA binding than do uninduced cells. We reported that addition of ^{3}H(-)t-7, 8-diol BP to tissue culture media resulted in the binding of metabolites of this compound to intact cellular DNA. In all cases the addition of epoxide hydrolase to the incubation or tissue culture media reduced the amount of reactive metabolites binding to DNA.

Highly purified cytochrome P-450s from the liver of rats treated with either 3-methylcholanthrene (3MC), phenobarbital (PB) or β-naphthoflavone (BNF) were used in a reconstituted system to analyze BP metabolism by high pressure liquid chromatography. In this study, twelve metabolites of BP were separated. Six unknown metabolites were also observed. BNF and 3MC-type cytochrome P-450 shows that in both cases 7, 8-dihydrodiol is a major diol, 3-OH-BP and 9-OH-BP are major phenols and 1, 6-, and 3, 6-quinones are major quinones. This result suggests that 3MC and BNF induced largely the same forms of cytochrome P-450. However, PB-type cytochrome P-450 is of a different form. The differences in metabolism are striking. Thus, the P-450-3MC and P-450-BNF show a large preference for metabolism at the 1, 3-position 7, 8-position and the 6-position, whereas the P-450 PB prefers metabolism at the 4, 5-position. All forms of P-450 show similar preference for the 9, 10-position.

Significance to Biomedical Research and the Program of the Institute: Metabolism of ^{3}H(-)t-7, 8-diol by the cellular mixed function oxidase system and binding of reactive metabolites to added and intact cellular DNA are essential steps for the initiation of chemical carcinogenic action. The inhibition of DNA binding by addition of epoxide hydrolase indicates that epoxide hydrolase may play an important role in detoxification in living cells.

Recent availability of the different forms of microsomal cytochromes P-450 in purified form has made it possible to study BP metabolism using the individual forms of cytochrome P-450 in a reconsitituted system. The substrate positional selectivity and stereoselectivity of different forms of cytochrome P-450 may regulate the balance between activation and detoxification pathways of BP metabolism.

Proposed Course: To determine the production of diol epoxide I and II in the incubation medium by using a cellular mixed function oxidase system and measuring the binding of reactive metabolites to added and intact cellular DNA. To understand the role of human liver epoxide hydrolase in the activation and detoxification of ^{3}H(-)t-7, 8-diol BP. The second project allows us to study the unique positional specificities of different forms of cytochrome P-450. These may determine the balance between activation on detoxification pathways of PAH.

Publications:

Gozukara, E.M., Belvedere, G., Robinson, R.C., Deutsch, J., Coon, M.J., Guengerich F.P. and Gelboin, H.V.: The effect of Epoxide hydrolase on Benzo (a)pyrene diol epoxide hydrolysis and binding to DNA and mixed function oxidase proteins. Molecular Pharmacol. 19: 153-161, 1981.

Gozukara, E.M., Guengerich, F.P., Miller, H., and Gelboin, H.V.: Different patterns of benzo(a)pyrene metabolism by purified cytochrome P-450s from 3-methylcholanthrene, phenobarbital and β-naphthoflovone treated rats: Identification of the 4- and 5- phenols as new metabolites. Carcinogenesis (In Press).

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05109-02-LMC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Interaction of Chemical Carcinogens with the Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Bustin	Visiting Scientist	LMC	NCI
OTHER:	M. M. Seidman	Senior Staff Fellow	LMC	NCI
	P. D. Kurth	Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)

Department of Human Genetics Tel Aviv University, Israel

LAB/BRANCH

Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.9	OTHER: 0.3

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interaction of a chemical carcinogen with the genome of a target cell is studied by 1) examining the binding of benzo(a)pyrene to SV40 minichromosomes 2) examining the binding of benzo(a)pyrene to polytene chromosomes and 3) using antibodies produced by benzo(a)pyrene modified DNA.

The results indicate that 1) the packing of the cellular DNA into the nucleosomal conformation does not significantly affect the binding of the carcinogen to DNA. 2) The in vivo incorporation of benzo(a)pyrene adducts into polytene chromosomes do not reveal "hot spots" for carcinogen binding. 3) The binding of BPDE-1 to the genome can be visualized by immunofluorescence and by immunoelectron microscopy.

Project Description

Objectives: To study the mechanism of the interaction of chemical carcinogens with the genome. To understand the influence of the structure and packing of the chromatin fiber on the binding of a carcinogen to its target.

Background and Research Strategy: The interaction of a carcinogen with the genome of a target cell seems to be the critical event leading to transformation. While it is known that certain chemical carcinogens interact with various constituents of the genome, it is not clear how the structure of chromatin and chromosomes influences the binding of the carcinogen to its target.

A program designed to elucidate some facets of these questions has been recently initiated. The program involves three different, albeit interrelated, experimental approaches.

The first approach involves the use of the SV40 minichromosome as a well-defined model for the eukaryotic chromosome. The major question under consideration is whether all reactive nucleotides are uniformly accessible to the carcinogen. This major question can be subdivided into the following more specific questions: 1) Is there a "hot spot" for carcinogen binding? 2) Is there variability between types of sequences, i.e., coding vs. regulatory, transcribed vs. non-transcribed? 3) Is there variability between coding and non-coding strand accessibility? 4) Is there difference between nucleosomal or spacer DNA?

The SV40 chromosome is an ideal system to study these questions since it is a well-defined system whose DNA sequence has been established.

The second approach involves the use of polytene chromosomes. These giant chromosomes are a convenient system to study various structure-function relations in the genome. Specific genes can be visualized by conventional light microscopy and the chromosomes can be experimentally manipulated so as to induce and visualize transcriptional activity in defined genetic loci.

While the SV40 system allows investigation at the DNA sequence level, the polytene chromosome system allows investigation of similar questions, but on a more macroscopic level. The following questions will be investigated: 1) Are there "hot spots" for carcinogen binding? 2) Does the carcinogen bind to a few selected genes? 3) Does a change in the macromolecular structure of a genetic locus affect the binding? 4) Is there a difference in the accessibility of the carcinogen between transcribed, condensed and uncondensed regions of the chromosome?

The third approach involves the use of antibodies specific for BPDE-I DNA to study various aspects of damage to the genome induced by the carcinogens and its subsequent repair.

Methods Employed: An encapsidation mutant growing in permissive cells is used as the target for the carcinogen. The carcinogen is either benzo(a)pyrene or its metabolite, benzo(a)pyrene 7, 8-diol-9, 10-epoxide I. Detection of the binding of the carcinogen to DNA is accomplished by two methods. The first is to use benzo(a)pyrene as a depurinating reagent and use accepted DNA sequence techniques to pinpoint the location of the damaged base. The second involves transfer of the modified DNA fragment to DBM paper and visualize the location of the modified DNA fragment by accepted immunochemical techniques.

For the polytene chromosome system, salivary glands are obtained from the 4th instar of Chironomus thummi. Polytene chromosome squashes are prepared by conventional techniques. The squashes are reacted with ^{3}H-diol epoxide I and the location of bound carcinogen visualized by autoradiography. The grain density in the various regions of the polytene chromosomes is determined semi-automatically using an Artek Model 880 micro/macro counting system.

For determination of the in vivo binding sites, larvae are grown in petri dishes in 10% DMSO and about 1 mCi ^{3}H-benzopyrene. After 1-24 hour incubation the larvae are washed and squashes of salivary glands are processed for autoradiographs.

The presence of a mixed-function oxidase system in Chironomus is detected by difference spectroscopy and by fluormetric assay for aryl hydrocarbon hydroxylase activity.

The ability of Chironomus to metabolize benzo(a)pyrene is determined by high performance liquid chromatography using a Whatman Partisil ODS-2 column with a linear 60-100% methanol-water gradient.

Major Findings: 1) An immunochemical method which allows the detection of one carcinogen adduct per SV40 genome has been developed. The technique allows localization of the carcinogen on the genome with the same precision as that obtained by analyzing DNA restriction sites and restriction fragments. Using this technique, we have compared the relative amount of carcinogen bound to the non-chromosomal regulatory region of SV40 to the amount bound to other SV40 fragments which presumably are in the nucleosomal conformation. A slight enhancement of BP binding to the origin of replication of SV40 as compared to the other sequences was detected. Since the enhancement is not more than two-fold, it is felt that it is not biologically significant. It is therefore concluded that the packing of the DNA into the nucleosomal conformation does not significantly influence the binding of the carcinogen to the genome. 2) The binding of the metabolites of BP to the giant chromosomes of Chironomus thummi can be visualized by autoradiography of chromosomes isolated from larvae which have metabolized ratioactive BP. So far the results suggest that the carcinogen binds to the chromosome in a random manner. Since the metabolities of BP bind to proteins, RNA and DNA, it is difficult to conclude that, at the DNA level, there are no preferred sites of interaction. The binding of the carcinogen to the DNA can be distinguished from the binding to proteins and RNA by using antibodies to BP-DNA adduct. A comparison of immunofluorescence with autoradiography will reveal whether there are preferential interaction sites at the DNA level. 3) Antibodies against BP-DNA were elicited in rabbits. Immunofluorescence studies indicate that the antibodies bind specifically to nuclei of cells treated with BPDE-I. A comparison of the fluorescence levels of various cells present on a slide revealed that the cells were modified to the same degree. Removal of the carcinogen and subsequent examination by immunofluorescence revealed that various cells in a culture have different abilities to remove the carcinogen. The location of BP metabolites on the DNA of the plasmid Col E-1 was visualized by immunoelectron microscopy.

Significance to Biomedical Research and the Program of the Institute: Understanding the processes involved in the interaction of chemical carcinogens with the genome of a target cell may help elucidate various aspects of the mechanism of carcinogenesis. The approaches used may be generally applicable to studies on the damage to cells induced by carcinogens and its subsequent repair.

Proposed Course: Studies aimed at accomplishing the goals of this project will be continued using the experimental approaches described above.

Publications

Seidman, M. M., Slor, H. and Bustin, M.: Immunological detection of carcinogen modified DNA fragments separated by agarose gel electrophoresis. J. Supramol. Structure, Supl. 5: 177, 1981.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05125-02 LMC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Study of Multiple Forms of Rat Microsomal Cytochrome P-450 by Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sang S. Park	Visiting Associate	LMC	NCI
OTHERS:	Harry V. Gelboin	Chief	LMC	NCI
	Tadahiko Fujino	Expert	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.8	PROFESSIONAL: 0.8	OTHER: 1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytochrome P-450, one of the key components of the mixed-function oxidase system has been found in multiple forms following induction by specific inducers. Hybridomas were prepared by fusion of myeloma cells with spleen cells of mice immunized with 3-methylcholanthrene-treated rat cytochrome (MC-P-450). Thirty-seven clones among 66 independent hybrid clones obtained in a selective medium produced monoclonal antibodies (MAB) to MC-P-450. Five of the six hybrid clones tested produced IgG1 in culture and the sixth clone produced IgM. The MAB found are specific for MC-P-450 with respect to protein binding, precipitation and inhibition of metabolic activity. The MABs interacted with β-napthoflavone induced cytochrome P-450, as well as with MC-P-450, but not with phenobarbital-induced cytochrome P-450. The MAB to MC-P-450 exhibited very similar inhibitory activity toward both the mixed-function oxidase and 7-ethoxycoumarin deethylase of microsomes. The MABs will be very useful for identification of multiple forms of cytochrome P-450 and for investigation of individual differences in susceptibility to chemical carcinogens.

Project Description

Objectives: Benzo(a)pyrene is activated by aryl hydrocarbon hydroxylase and epoxide hydratase which exist in multiple forms. Metabolism of BP by these enzymes leads to both directions: to the formation of nontoxic water-soluble conjugates and/or to the formation of the active carcinogenic forms of BP, BP-7, 8-diol-9, 10-epoxides. Any change in cell environment might shift the balance of the metabolism to produce different proportions of these metabolic products. Rat liver microsomes are highly inducible for the carcinogen metabolizing enzymes. We use this rat system as a model for the study of the nature of human carcinogen metabolizing enzymes.

Methods Employed: Balb/c female mice were immunized with purified cytochrome P-450 of rats which were treated with 3-methylcholanthrene. The primed spleen cells were isolated and fused with myeloma cells using polyethylene glycol. The hybrid cells were grown in a selective medium (HAT) and screened for hybridoma-producing monoclonal antibodies to MC-induced cytochrome P-450 by radioimmunoassay.

Major Findings: (1) Thirty-seven clones among 66 independent hybrid clones produced monoclonal antibodies to MC-P-450. The immunoglobulins produced are types of IgG1 and IgM. The monoclonal antibodies are specific for MC-P-450 with respect to protein binding, precipitation and inhibition of metabolic activity. (2) The monoclonal antibodies interacted with another P-450 isozyme, -naphtoflavone induced P-450, as well as with MC-P-450, but not with phenobarbital-induced cytochrome P-450. (3) The monoclonal antibodies also inhibit the aryl hydrocarbon hydroxylase activities of placental microsomes of women who smoke heavily.

Significance to Biomedical Research and the Program of the Institute: Metabolic balance of benzo(a)pyrene between detoxification and carcinogenesis is dependent on the presence of a specific, predominant form of multiple enzymes of individuals exposed to different environments. Monoclonal antibodies made against MC-induced cytochrome P-450 are specific to the MC-P-450 and cross-react with -naphtoflavone induced cytochrome P-450, but not phenobarbital induced P-450. Therefore, it appeared that a typical cytochrome P-450 could be induced by several inducers with common properties.

Proposed Course: (1) Monoclonal antibodies to MC-induced rat cytochrome P-450 inhibited the enzyme activity of human placenta microsomes with the indication that the monoclonal antibodies might cross-react with one of the human placenta microsomal cytochrome P-450's which is inducible by carcinogens. (2) The cross-reactivity of the monoclonal antibodies will be tested with cytochrome P-450 from rats, rabbit and human materials. (3) For the purpose of developing screening procedures for individuals prone to chemical carcinogensis the monoclonal antibodies will be labeled with ^{125}I and fluorescein, and radioimmunoassay and immunofluorescence techniques will be developed in order to determine the levels of enzymes involved in BP metabolism.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05195-01 LMC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetics and Regulation of Cytochrome P-450 Biosynthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. B. Fagan	Senior Staff Fellow	LMC	NCI
OTHER:	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.7	0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytochrome P-450 is a central component of the aryl hydrocarbon hydroxylase (AHH) system. Regulation of AHH enzymatic activity has been carefully studied in vivo, as well as in cell culture using buffalo rat liver (BRL) cells and other cell types. The accessibility of the BRL cell culture system to well-controlled experimental manipulation makes it an ideal system for studying the regulation of biosynthesis of the cytochromes P-450 and of other AHH components. There is no direct information on the regulation of synthesis, processing and turnover of AHH-system polypeptides, nor is there direct information on the regulation of rates of transcription, processing and degradation of the mRNAs for AHH-system enzymes. Our objective is to use the BRL cell culture system to study regulation of the AHH system at these two levels.

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Project Description

Objectives:

1) Select clones of BRL cells which are highly inducible for AHH and are of diploid or near diploid karyotype. These cells will be used for all subsequent studies.

2) Characterize the rates of P-450 synthesis in control and polycyclic hydrocarbon (PAH) induced BRL cells.

3) Characterize the stability and half-life of P-450 polypeptides in control and PAH induced BRL cells.

4) The enzymatic activity of BRL cells has been shown to be inducible by a number of different treatments. In order to understand the underlying mechanisms of these effects, we will characterize the effects of these treatments on P-450 polypeptide synthesis, processing stability and turnover. The treatments to be studied include cultures with PAHs, temporary inhibition of protein synthesis, culture with cyclic AMP and culture with butyrate.

5) Compare the extents and time courses of induction of P-450 peptide synthesis with previously reported data on the effects of inducers on AHH enzymatic activity.

6) Study processing (by proteolysis, glycosylation and heme-insertion) of newly synthesized P-450 peptides.

7) Objectives 2 through 8 will also be carried out for the regulation of other enzymatic components of the AHH system including epoxide hydratase (EH).

8) Study the regulation of synthesis, processing and degradation of mRNAs for AHH-system proteins. This work is contingent upon the construction and isolation of cloned cDNAs complimentary to the P-450s and other AHH enzymes (see annual report Z01-CP-05196-01-LMC). Establishment of specific objectives and experimental design are also contingent upon the understanding of regulation of peptide synthesis, which will come out of the work outlined in objectives 2 through 7. For these reasons, detailed objectives at the mRNA level will be deferred to future reports.

9) Isolate BRL derivatives lacking basal and inducible AHH activity. Biochemical and molecular biological analysis of variant BRL lines lacking basal and/or induced AHH activity will be useful in analyzing the regulation of the AHH system. It should be possible to obtain mutants in all components of the system, since the AHH system is not essential for cell surival in culture. Mutants in reductase, P-450s, EH, the transferases, and in the PAH receptor protein should be isolatable. Other classes of mutants, such as regulatory mutants (in addition to receptor protein-type regulatory mutants) may also be identified.

Comparisons of carcinogenicity, mutagenicity and cytotoxicity of various PAHs in a series of cell lines lacking various components of the AHH system would be useful in evaluating the roles of these components in rendering PAHs carcinogenic, mutagenic and cytotoxic.

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Methods Employed: Standard cell culture, karyotyping and cloning procedures are being used with BRL cells. Rates of peptide synthesis, stability, turnover and degradation are measured by labeling cells in culture with ^{35}S-methionine (either steady state or pulse-chase labeling). This is followed by immunoprecipitation of solubilized, ^{35}S-labeled peptides and analysis and quantitation by SDS-polyacrylamide gel electrophoresis. Regulation of synthesis, processing and degradation of AHH system mRNAs will be studied using the procedures outlined in annual report Z01-CP-05196-01-LMC. BRL variants, lacking various components of the AHH system, will be isolated using two different procedures. Variants which are unable to metabolize PAHs will be identified by fluorescence-activated-cell-sorting of cells cultured in the presence of a PAH such as benzanthracene, whose fluorescence spectrum changes when it is metabolized. AHH negative variants will also be selected by growth in the presence of a PAH which is relatively nontoxic until it is metabolized via the AHH system.

Major Findings: 1) Culture of BRL cells in the presence of benzanthracene (BA) for 12-18 hours causes a 4-to 8-fold increase in steady state labeled peptides which are immunoprecipitated with IgG specific for highly purified MC-type P-450. 2) the rate of synthesis of peptides immunoprecipitible with MC-P-450-specific IgG is linear for about 60 minutes, and this rate is increased in cells induced with BA. 3) Two immunoprecipitible peptides of about 55 and 57 kilodaltons are induced by BA. 4) Tunicamycin, an inhibitor of peptide glycosylation, blocks the accumulation of ^{35}S-labeled peptides immunoprecipitated with MC-P-450 IgG.

Significance to Biomedical Research and to the Program of the Institute: The AHH system is central to the activation and detoxification of carcinogens, to the detoxification of xenobiotics and to drug metabolism. A wealth of evidence indicates that the AHH system is very sensitively regulated in vivo. However, the molecular mechanisms of this regulation have not yet been elucidated. The significance of this regulation, especially the induction of specific P-450 species, in determining the response of individuals to specific carcinogens, drugs and xenobiotics has not been assessed. Accomplishment of the objectives described above should provide new insights into the molecular mechanisms of regulation of the biosynthesis of the enzymatic components of the AHH system. Furthermore, comparisons of the abilities of a series of cell lines lacking various components of the AHH system to convert PAHs to carcinogenic and cytotoxic forms will be useful in evaluating the roles of those components of the AHH system in rendering PAHs carcinogenic and/or cytotoxic. Accomplishment of these objectives will result in a better understanding of the balance between activation and detoxification of carcinogens, the influence of this system on drug action and other questions in carcinogensis, toxicology and pharmacology. It also should be noted that the properties of the BRL system make it an extremely useful model system for studying the regulation of eukaryotic gene expression. Thus, this work should not only contribute in a practical way to the areas of carcinogenesis, toxicology and pharmacology, but, as well, will contribute to the basic understanding of eukaryotic gene regulation.

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Proposed Course: Objective 1 has been attained and work is in progress and some findings have been attained for objectives 2 through 7. In this upcoming research period, these objectives, as well as the other objectives, will be pursued.

Publications:

None

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05196-01 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cloning, Structure and Regulation of the Genes for the Cytochromes P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. B. Fagan	Senior Staff Fellow	LMC	NCI
OTHER:	H. V. Gelboin	Chief	LMC	NCI
	E. Gozukara	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	0.8	1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cytochromes P-450 are central to the activation and detoxification of xenobiotics, to drug metabolism and to the activation and detoxification of carcinogens. Whether a potentially carcinogenic molecule is metabolized to an active carcinogen or to an excretable form may depend on the activity and substrate specificity of the forms of the P-450s which predominate in the target tissue. As a result of this, susceptibility to cancer induction may well depend on the regulation of expression of the genes for specific cytochrome P-450. We are using recombinant DNA techniques and other state-of-the-art molecular biological methodologies to study the structure and the regulation of the genes for the P-450s. We have constructed cDNA clones complementary to methylcholantrene induced P-450 mRNA, and are constructing equivalent cDNA clones complimentary to phenobarbital-induced P-450 mRNA. These cDNA clones are being used to clone the complete, native genes for these P-450s. We will use these recombinant molecules to study the structure of the P-450 genes and the regulation of expression of these genes.

Project Description

Objectives:

1) Construct, identify and characterize clones of cDNA complementary to the mRNAs for the cytochromes P-450 induced by methylchlanthrene, phenobarbital and other inducers.

2) Isolate and characterize cloned fragments of the rat genome containing the native genes for inducible cytochromes P-450.

3) Study regulation of P-450 gene expression at the mRNA level using cloned P-450 cDNA and gene sequences as hybridization probes. Specific objectives: a) Quantitation of the levels of P-450 mRNAs under various induction conditions. b) Quantitation of rates of transcription of P-450 mRNAs. c) Quantitation of rates of degradation of P-450 mRNAs. d) Characterization of rates and routes of processing of P-450 mRNAs.

4) Analyze the structure of the P-450 genes. Specific objectives: a) Detailed characterization of the regulatory sequences associated with the P-450 structural genes by DNA sequence analysis of the cloned P-450 genes. b) Electron microscopic analysis of heteroduplexes between P-450 mRNA and cloned P-450 genes to assess splicing and other structural characteristics of the P-450 mRNAs and genes. c) Comparison of the regulatory sequences associated with different P-450s that are induced by different compounds. d) Comparison of the structural genes for P-450s induced by different compounds.

5) Correlate regulatory data from objective 3 with structural data from objective 4 for the purpose of identifying relationships between gene structure and regulation.

6) Use the P-450 cDNA and genomic clones to assess a) the multiplicity of P-450s expressed in different tissues; b) the relatedness of P-450s expressed in different tissues; and c) the relatedness of P-450 genes in different eukaryotes. Both the regulatory and structural sequences will be assessed.

Methods Employed: Standard methods (as previously reported in Fagan *et al*., *J. Biol. Chem.* 256: 520-525, 1981 and Fagan *et al*., *Nucleic Acids Res* 6: 3471-3480, 1979) for RNA isolation, fractionation and characterization have been modified, where necessary, for use with rat liver. These include RNA extraction and isolation with guanidine-HCL, RNA size-fractionation by methylmercuric hydroxide agarose gel electrophoresis and by sucrose gradient centrifugation, *in vitro* translation of mRNA, immunoprecipitation of translation products with P-450 specific IgG and SDS-polyacrylamide gel electrophoretic analysis of translation products and immunoprecipitates. Levels and rates of RNA synthesis will be studied by Northern blot hybridization and solution hybridization to P-450 cDNAs. DNA complementary to P-450 mRNA is being synthesized and cloned in the plasmid pBR322 by modifications of previously reported methods (see reference above). Clones of the complete P-450 genes will be isolated from libraries of rat genomic DNA ligated into phage λ.

Clones will be identified by standard placque hybridization techniques. Cloned cDNAs and genes are prepared in large amounts by standard microbiological and biochemical procedures. Cloned cDNAs and genes will be characterized and studied by a wide range of techniques including heteroduplex mapping, hybridization selected translation and immunoprecipitation, restriction endonuclease analysis, rapid DNA sequencing, southern blot hybridization, solution hybridization, and *in vitro* transcription analysis.

Major Findings: To date we have 1) cloned cDNAs complementary to MC-P-450 in plasmid pBR322; 2) used these cloned cDNA sequences to quantitate MC-P-450 mRNA levels in control and MC-induced rat liver; and 3) by two independent methods we have determined that there are at least two different sizes of MC-inducible mRNA that code for peptides immunoprecipitible with IgG highly specific for MC-P-450. These mRNAs are 1700-1900 bases and 2400-2600 bases in length. They code for immunoprecipitable peptides of different sizes.

Significance to Biomedical Research and the Program of the Institute: The P-450s, as part of the mixed-function oxidase system, are central to the activation and detoxification of carcinogens, to the detoxification of xenobiotics and to drug metabolism. There is a wealth of evidence indicating that these enzymes are highly inducible and that they exist in multiple forms. The source of the diversity of P-450s and the mechanisms of their regulation have not yet been elucidated at the molecular level. The significance of the diversity and regulation of the P-450s in determining the response of individuals to specific carcinogens, drugs and xenobiotics has not been assessed. Accomplishment of the objectives described above should result in new insights into the molecular basis for the diversity of P-450s and into the molecular mechanisms of regulation of P-450 gene expression. This will result in a better understanding of the balance between activation and detoxification of carcinogens, the influence of this system on drug action and other questions in carcinogenesis, toxicology and pharmacology. Furthermore, since the P-450 system is inducible and is highly accessible to experimental manipulation both *in vivo* and in cell culture, this system is also interesting on a more fundamental level as a model system for studying the regulation of expression of a family of genes. Thus, the work with this system should contribute to the basic understanding of eukaryotic gene regulation.

Proposed Course: We presently have cDNA clones for MC-P-450; the isolation and characterization of cDNA for other P-450s is in progress. Isolation of genomic clones will be carried out in the upcoming research period, and analysis of P-450 gene structure will follow isolation of genomic clones. We have begun to study P-450 mRNA levels; this will be continued along with analysis of P-450 mRNA transcription, processing and degradation.

Publications:

Fagan, J.B., Pastewka, J.V., and Gelboin, H.V.: Recombinant bacterial plasmids with sequences complementary to methyl-cholanthrene induced cytochrome P-450 mRNA: construction and identification. *J. Biol. Chem.* 1981 (In Press)

Fagan, J.B., Pastewka, J.V., Guengerich, F., and Gelboin, H.V.,: Multiple forms of inducible cytochromes P-450 are translated from different mRNAs. Nucleic Acids Research (In Press)

Fagan, J.B., Park, S.S., Guengerich, F., and Gelboin, H.V.,: DNA cloning and monoclonal antibodies: analysis of cytochrome P-450 regulation. Proc. Fifth International Symposium on Microsomes a Drug Oxidases. Jap. Sci. Soc. Press, (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05197-01 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
DNA Repair Capacity in Human Tumor Cell Strains

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Daniel B. Yarosh	Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Most human tumor cell strains are capable of supporting the growth of adenoviruses which have been treated with carcinogenic compounds such as N-methyl-N'-nitro-N-nitrosoguanidine and methylnitrosourea which alkylate DNA. About one-fifth of the cell strains tested are significantly deficient in supporting growth of the damaged viruses. We interpret this as a deficiency in DNA repair. The ability of cell strains to recognize and incise alkylated DNA are studied in extracts of cells acting on bacterial plasmid DNA alkylated in vitro. A technique to measure this incision using DNA affinity chromatography has been developed. The kinetics of incision by extracts of cells are being studied. In addition, we are studying repair replication in cell strains following treatment with DNA alkylating agents, using the technique of photolysis of bromodeoxyuridine (BUDR) incorporated during repair.

Project Description

Objectives: To study DNA repair capacities in cell strains derived from human tumors. To identify repair deficiencies, if any, and to characterize them on a molecular level. To determine if any DNA repair deficiencies suggest potential therapeutic approaches for cancer patients whose tumor cells show DNA repair deficiencies. To evolve a screening procedure to identify patients whose tumors might best respond to treatments based on DNA repair deficiencies in the malignant cells.

Methods Employed: Many assays for DNA repair capacity are currently used. Two new techniques are a DNA incision assay using DNA affinity chromatography and photolysis of BUDR incorporated during repair. The DNA incision assay measures the presence of activities in extracts of tumor cells which incise DNA treated with alkylating agents but not untreated DNA. Incision of DNA is measured using radioactively labeled supercoiled plasmid DNA as a substrate for the activity in tumor cell extracts. Incision of plasmid DNA converts it from the supercoiled state to a relaxed state and these can be separated by their affinity for acridine yellow dye bound to bis-acrylamide. Because acridine is an intercalating agent, supercoiled DNA is preferentially bound and is eluted from a column at a higher salt concentration than is relaxed DNA. The BUDR photolysis assay measures the increase in sensitivity to 313 nm light of DNA substituted with BUDR. Tumor cells are treated with alkylating agents and allowed to repair in the presence of BUDR. The amount of excision of lesions and repair synthesis can be determined from the increase in breaks induced by 313 nm light in DNA repaired with BUDR compared to DNA repaired with thymidine.

Major Findings: 1) A project begun prior to this year was completed during the year. This work used the BUDR photolysis assay to measure the average patch size of excision repair of UV lesions in bacteriophage T4. The results show that on the average phage T4 reinserts four nucleotides for every pyrimidine dimer excised. 2) Extracts from four human tumor cell strains were shown to contain activities which recognize and incise alkylated DNA. 3) An extract from a human tumor cell strain which is deficient in support of alkylated adenovirus was shown to be deficient in any activity which incises alkylated DNA. 4) A human tumor cell strain proficient in support of alkylated adenovirus was shown to incorporate BUDR during repair synthesis, while a human tumor cell strain deficient in support of treated adenovirus failed to incorporate nucleotides during repair.

Significance to Biomedical Research and the Program of the Institute: These results suggest that *in vitro* tests of DNA repair capacity may be diagnostic of the ability of cells to respond to carcinogens. This implies that tumors may arise from cells in which DNA repair capacity has been impaired by somatic mutation. It also suggests that some cancers may respond to treatment with drugs against which the cancer cells have lost their ability to recover successfully.

Proposed Course: 1) To characterize the DNA repair systems which act on alkylated DNA in human cell strains. 2) To identify the molecular defect in human tumor cell strains which are deficient in support of alkylated adenovirus. 3) To measure mutagenicity in alkylated DNA after repair in proficient and deficient human tumor cell strains.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05200-01 LMC

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Molecular Mechanism of Mutation Generated by Environmental Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakefuda	Medical Officer	LMC	NCI
OTHER:	H. Mizusawa	Visiting Scientist	LMC	NCI
	Che-Hung R. Lee	Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)

M. Rosenberg, LB, NCI, NIH
K. McKenney, LB, NCI, NIH
H. Shimatake, LB, NCI, NIH

T. Sugimura, Director and M. Nagao, Biologist, National Cancer Research Institute, Toyko, Japan.

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The topic of the present study is the establishment of a plasmid-mediated mutation assay system which allows us to determine changes in DNA sequence. These changes are directly correlated with the mutagenized function of a specific gene.

Pretreatment of plasmid DNA with carcinogen prior to transfection into E.coli permits us to assay mutation with no toxic effect from drugs.

Alternatively, the plasmid can be transfected into E.coli followed by exposure to carcinogen with or without the microsome fraction of rat liver depending on the drugs' requirements for metabolic activation.

Mutated plasmid DNAs isolated and cloned individually are investigated for determination of the chemical nature of DNA modification. The altered genetic functions of mutagenized plasmid DNA are tested by repeated transfection of the DNA into E.coli.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: 1) To observe the molecular mechanism of mutation generated by chemical carcinogens; 2) to observe the genetic factors of the cell involved in mutagenesis; 3) to observe the structure/function relationship of mutation at a single DNA base level.

Methods Employed: 1) The benzo(a)pyrene (BP) metabolite, trans-7,8-diol- 9,10-epoxide (BPDE-I) and closed circular DNA of plasmid pK0482 were incubated for DNA modification. The rate of modification was measured by the radioactivity of the bound BPDE-I. The pK0482 plasmid consisted of galactose kinase (gal K) and B-lactamase (amp-r) structural genes. 2) The pKG1820 plasmid consisted of a transcriptional terminator sequence between the promoter and gal K gene. 3) *E. coli* strains (gal K^-) of AB 1886 ($uvrA^-$), AB2463 (rec A^-) and AB1157 (wild type) were transfected with BPDE-I modified or unmodified pK0482 DNA. Transformants were selected by the acquired ampicillin resistancy and mutated colonies were counted on MacConkey galactose (1%) agar plates containing ampicillin (50 ug/ml). 4) In other experiments, the *E. coli* strains were transfected with intact pKG1820 plasmid DNA first and exposed to either BPDE-I or mutagenic tryptophan pyrolysate (Trp-P-1) with S-9 fraction of rat liver microsome which was previously induced by PCB. 5) The plasmid DNA was isolated from colonies which showed mutated phenotype and repeatedly transfected in *E. coli* to obtain stable mutated DNA. 6) The DNA fragment containing the terminator was isolated from mutated pKG1820 DNA by restriction enzyme treatment and gel electrophoresis and was sequenced by Maxam and Gilbert's method.

Major Findings: In AB1157 (wild type) strain, a transformation efficiency of 37% was observed when approximately three molecules of BPDE-I were covalently bound to a single pK0482 DNA. In AB1886, ($uvrA^-$) a 37% transformation efficiency was produced by only one molecule of BPDE-I per molecule of pK0482 DNA. The mutation frequency in the $uvrA^-$ strain was significantly higher than the wild type suggesting that BPDE-I binding in the unexcised plasmid DNA resulted in a higher rate of mutation. In AB2463 ($recA^-$) strain, the transformation efficiency was similar to that with AB1157, but mutagenesis was suppressed. The expression of the gal K was blocked by the terminator sequence inserted artificially between the promoter and gal K structure genes in pKG1820 DNA. This blockage was obviated by either (1) mutation generated at the terminator or (2) frameshift mutation occurring 100 bases upstream of the amber codon, which is essential for translation terminator. In the present experiments, colonies which showed expression of the gal K gene (as the result of defective terminator caused by mutation) were picked and the plasmid DNAs were further purified by single colony isolation. The sequencing of terminator region showed an insertion of an A:T pair in the $(A:T)_6$ cluster or deletion of the (G:C) pair from the $(G:C)_3$ cluster. All of these were frameshift mutations, suggesting that the cluster regions are "hot spots" in terms of mutagenesis by BPDE-I. Such insertion or deletion of a base-pair has never been observed in the control plasmid DNA. A similar experiment was carried out with tryptophan pyrolysate, TRP-P-1 mixed with rat microsomal fraction and host strains of *E. coli*. An identical defect in the terminator function by mutation in pKG1820 was observed. The sequencing of the DNA is in progress.

Significance to the Biomedical Research Program of the Institute: (1) Carcinogen-DNA interaction is an important initial step for mutagenesis and carcinogenesis. Since many mutagens are carcinogens, effective assay methods for screening of mutagens are of considerable importance for detection of potential environmental carcinogens. The plasmid mediated mutation assay, developed in our laboratory, allowed us to assay mutation entirely free from the toxic effect of muta-carcinogens, the majority of which are usually potent toxic agents. Thus, the frequency of mutation could be increased up to 10^{-2} level with highly toxic BPDE-I. (2) The molecular mechanism of mutation could be analyzed by sequencing of the DNA segment, the genetic function of which is known. The structure/function relationship becomes clear by this plasmid-mediated mutation assay.

Proposed Course: The plasmid-mediated mutation assay developed in our laboratory, will continue to be applied for a number of other environmental carcinogens. Involvement of SOS repair function on the generation of mutation will be tested by UV preirradiation of the host cells prior to transfection of plasmid DNA. A new recombinant DNA consisting of structural gene of metallothionein (MT) will be constructed, propagated in *E. coli*, and transferred into SV40 vector. The expression of the MT gene in African green monkey kidney cells is monitored by acquired resistancy to toxic metals. Mutation by chemical carcinogens generated at the MT structural gene or regulatory gene will be analyzed by DNA sequencing.

Publications:

Mizusawa, H., Lee, C-H., Kakefuda, T.: Alteration of plasmid DNA-mediated transformation and mutation induced by covalent binding of benzo(a)pyrene-7, 8-dihydrodiol-9,10-oxide in *Esherichia coli*. *Mutation Res.* (In Press)

Slor, H., Mizusawa, H., Neihart, N., Kakefuda, T., Day, R.S. III, and Bustin, M: Immunochemical visualization of the binding of a chemical carcinogen to the genome. *Cancer Res.* (In Press)

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05201-01 LMC

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies on the Dynamic Changes of DNA Helix Associated with Carcinogen Interactions.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakefuda	Medical Officer	LMC	NCI
OTHER:	C-H. R. Lee	Staff Fellow	LMC	NCI
	H. Mizusawa	Visiting Scientist	LMC	NCI

COOPERATING UNITS (if any)
R.J. Feldman, DCRT, NIH
T. Sugimura, Director, National Cancer Center Res. Inst., Tokyo, Japan
M. Nagao, Biologist, National Cancer Center Res. Inst., Tokyo, Japan

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The topic of the present study is the accumulation of new information regarding the structural modification of the DNA helix associated with carcinogen interactions. A few model systems have been established to analyze the conformational change of DNA resulting from covalent and non-covalent binding of carcinogenic drugs, dehydration by organic solvents, polyamines, and mutagenic amino acid pyrolysates. Stereospecificity of carcinogen interaction depends in part on the conformation of the DNA helix. Alteration of target sites of DNA structural components associated with covalent interaction with carcinogens are studied under a variety of physico-chemical experimental conditions.

Project Description

Objectives: 1) To observe different forms of structural modifications of DNA by carcinogen interactions; 2) to observe the dynamic change of the DNA helix resulting from the microenvironmental changes (physical and chemical) surrounding the helix; 3) to make antibodies against carcinogen DNA adducts and to determine the geometry of DNA modification on DNA as well as in the cells; 4) to make available information obtained from basic experimentation for the understanding of the structural organization of the genetic components of living organisms.

Methods Employed: 1) Covalently closed DNAs (pBR322 or SV40) with different superhelical densities were produced by incubating linearized (by endonuclease EcoR1) DNAs with T4 ligase in the presence of different kinds of organic solvents, salts, polyamines, putrescine, and cadaverine at various temperatures. The DNAs were precipitated by ethanol and analyzed by two-dimensional agarose gel electrophoresis, adding ethidium bromide only in the second electrophoretic run. 2) A similar experiment, using DNA relaxation enzyme instead of ligase, was carried out in the presence of a variety of benzo(a)pyrene metabolites, and muta-carcinogenic amino acid pyrolysates to observe the noncovalent interactions with DNA. 3) Equilibrium dialysis, UV absorption spectroscopy and fluorescence quenching were carried out to find DNA-carcinogen interactions. 4) An oligonucleotide with three DNA base-pairs was produced by computer graphic technology. Graphic models of B(a)P metabolites and its hydrolysis products, psoralen, amino acid pyrolysates, etc., were also constructed. Crystallographic atomic coordination parameters, energy calculation and physical space filling models were used for input information for computation. The interactions and other interactions between the DNA and drugs were observed by stereo projection on a television screen. These were photographed and atomic and molecular configurations were analyzed. 5) Circular dichroism spectroscopy was taken to analyze the conformational changes of DNA which were bound with various muta-carcinogenic drugs. 6) Antisera specific to DNA modified with BPDE-I was produced and reacted with DNA. Indirect immunofluorescent and immuno-electron microscopy were also carried out.

Major Findings: 1) The two-dimensional gel electrophoresis of DNA ligated in the presence of organic solvents or at different temperatures showed that a) the DNA helix unwinds upon dehydration by organic solvents or elevation of temperature; b) the conformation change is a continuous process; c) the H of unwinding one linking of DNA helix is constant at about 12.2 ± 0.4 kcal/mole; the corresponding S and d(S)/dn are 2nkR and 2kR (n = relative equivalent linking number; R = gas constant, k = 1117/number of base-pairs); d) the d(S)/dn, like k, is inversely proportional to the number of base-pairs; e) the double-stranded DNAs of different chain length have average S = 35 cal/mole °C for unwinding one linking and this corresponds to 127 ± 14 base-pairs per "relative linking" number. 2) Covalent binding of BPDE-I to DNA of less than 0.2% did not significantly alter the superhelical density of supercoiled circular DNA as detected by gel electrophoresis. An increased amount of covalent binding caused cumulative microenvironmental change in the minor groove of the DNA helix, such as dehydration as described above. This provided a new target site for interaction which was normally unreactive or poorly reactive with BPDE-I. 3) B(a)P with hydroxylation at the 7,8 position (7,8diol, 7,8,9,1 0-tetrol and 7,8,9-triol B(a)P intercalated DNA. Intercalation of BPDE-I covalently bound to guanine residue is, however, unlikely to occur because of highly restricted molecular orientation. 4) The antisera specific to DNA modified with BPDE-I reacted with either single-stranded or nature-modified DNA, but not with unmodified DNA. It did not react with free BPDE-I or with proteins modified by BPDE-I. The immuofluorescence study indicated that the IgG in the sera bound specifically to the nuclei of human fibrobast in culture. The intensity of fluorescence was proportional to the dose of BPDE-I to which the cells were exposed. About 50% of the BPDE-I adducts remain bound to DNA 24 hours after the removal of carcinogen. An electron microscopic observation of DNA reacted with ferritin conjugated IgG revealed that the ferritin molecules are exclusively located on the modified DNA. 5) Many active carcinogens, precursors and related compounds were found to have a relatively strong affinity to DNA as observed by DNA relaxation enzyme study, equilibrium dialysis, and fluorescence quenching. Those compounds and metabolites that are neither mutagenic nor carcinogenic showed a poor affinity to DNA, suggesting that the mechanism of muta-carcinogenesis involves physicochemical interaction with DNA in addition to metabolic activation.

Significance to Biomedical Research and Program of the Institute: Unwinding of the helix is a characteristic dynamic event associated with genetic functions of DNA, such as transcription and replication. Winding and unwinding of DNA occurs when the DNA template is read by polymerases or assembling of nucleosomes in the cell. Dynamic changes of DNA helix have been observed with intercalating agents, metallic ions, gyrase, DNA relaxation enzyme, and histone, etc. The analysis of DNA structure under different physico-chemical conditions provides highly useful information for our understanding of the macromolecular organization and the functional aspects of the genetic machinery in living systems. Another important aspect of DNA modification is the interaction between DNA and drugs, including carcinogens, mutagens, and chemotherapeutic agents. In our present study, a remarkably strong correlation of mutagenic potential and non-covalent interaction was found, suggesting that generation of mutation involves noncovalent binding of the compounds to DNA prior to metabolic activation and covalent binding. Such great affinity may be a necessary, but not sufficient, step for mutagenesis and possibly for carcinogenesis. A number of new methods developed in our laboratory allow us to analyze a single linking number difference generated by either dehydration, temperature, or drug interaction.

Proposed Course: Non-covalent and covalent interaction of many compounds will be tested and correlated with their mutagenic potential, which are in progress as a separate research project in our laboratory. Covalent binding of muta-carcinogens to different structural components (bases and phosphate group) under different experimental conditions which facilitates winding or unwinding of the helix will be studied using high pressure liquid chromatographic analysis of the adduct. Shifting of stereospecificity and target molecules in DNA for covalent binding are expected to occur depending on the DNA conformation.

Publications:

Kakefuda, T., Mizusawa, H., Lee, C-H., Madigan, P., Feldman, R.J.: Structural modifications and their effects on the genetic functions of DNA generated by interactions with benzo(a)pyrene metabolites. In Pullman, B., Ts'o, P.O.P., and Gelboin, H.V. (Eds.): Carcinogenesis: Fundamental Mechanisms and Environmental Effects. Dordrecht, Boston & London, D. Reidel Publishing Co., 1980, pp. 389-407.

Lee, C-H., Mizusawa, H., and Kakefuda, T.: The unwinding of double-stranded DNA helix by dehydration. Proc. Nat. Acad. Sci. (In Press)

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05204-01 LMC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
DNA Sequence Alterations In Vivo Following DNA Modification by Carcinogens.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael Seidman	Sr. Staff Fellow	LMC	NCI
OTHER:	Sekhar Chakrabarti	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A system has been developed to assay DNA sequence changes which arise in cells as a result of covalent modification of the DNA by chemical carcinogens. The program involves modification of a specific DNA fragment with the active form of benzo(a)pyrene, the 7, 8-diol, 9, 10-epoxide, followed by ligation of the fragment into an appropriate plasmid vector. The ligation products are introduced into cells, replicated and the progeny DNA molecules harvested and screened for changes in the sequence of the modified fragment. This protocol is designed to measure forward mutations and avoid the selective constraints of the standard back mutation mutagenesis assays. In addition, it will permit the distinction between sequence changes which arise directly from DNA modification and those which occur from error-prone replication induced by DNA damage at distal sites. Although the amplification and assay of mutagenic events occur in bacteria, appropriate vectors can be used for the same types of experiments in human cells followed by analysis in bacteria.

Project Description

Objectives: 1) To develop a system to assay DNA sequence changes which occur in vivo after covalent DNA modification by chemical carcinogens, 2) to distinguish between DNA sequence changes which arise directly from DNA modification and those which are the result of error-prone replication induced by DNA modification.

Methods Employed: A 600 base-pair fragment of DNA from the plasmid PBR322 is purified and modified covalently with the 7, 8 diol, 9, 10-epoxide form of benzo(a) pyrene. The fragment is then ligated back into the large PBR fragment derived from the double enzyme digestion which produced the 600 base-pair fragment. Correct ligation restores the information for tetracycline resistance to the plasmid. In addition, the 600 base-pair fragment contains several single cut enzyme sites. Thus, there are two assays for screening mutants: one based on tetracycline resistance/sensitivity, the other on the presence or absence of the single cut enzyme sites. The first assay is being used during the development of the system; the second will be used to collect all mutations at a given site regardless of phenotypic consequence. In either assay the final analysis will involve DNA sequencing.

Major Findings: The recipient cells must be induced for SOS response before transformation. Without this treatment the transformation frequency is very low and no mutants appear. With SOS induction the transformation frequency in several orders of magnitude higher and mutants can be acquired.

Significance of Biomedical Research and the Program of the Institute: The study should permit a direct assessment of the consequences of covalent modification of DNA by chemical carcinogens. The use of vectors appropriate for mammalian cells will provide an opportunity to assay, at the molecular level, the defects in cells from individuals with hereditary disorders such as xeroderma and Bloom's syndrome.

Proposed Course: The mutants obtained in the prokaryotic studies will be characterized and the protocol extended to human cells.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05205-01 LMC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of the Multiplicity, Diversity and Catalytic Properties of Cytochromes P-450 Using Specific Inhibitors of Different Forms of the Mixed-Function Oxidases.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H.V. Gelboin	Chief	LMC	NCI
OTHER:	D. West	Biologist	LMC	NCI

COOPERATING UNITS (if any)

T. Sugimura, National Cancer Research Institute
Tokyo, Japan

LAB/BRANCH

Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.2	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mixed-function oxidases containing different forms of cytochrome P-450 are the critical enzymes that metabolize a wide variety of drugs, chemicals and carcinogens. The focus of this project is the utilization of specific inhibitors and inducers of aryl hyrdocarbon hydroxylase (AHH) to probe the multiplicity, diversity and different catalytic properties of the cytochrome P-450s. The form of AHH that is induced by polycyclic hydrocarbons is strongly inhibited by 7, 8-benzoflavone, but this compound weakly inhibits or stimulates the form of AHH from untreated or phenabarbital-treated rats. In contrast, 1-Maackiain acetate greatly inhibits the AHH from untreated or phenobarbital-treated rats, and weakly inhibits or stimulates the form of AHH induced by polycyclic hydrocarbons. Therefore, 7, 8-benzoflavone and 1-Maackiain acetate are inhibitors that are effective against different forms of AHH. We are using these inhibitors to probe the enzymology and active catalytic sites of the multiple forms of the cytochromes P-450.

Project Description

Objectives: To use specific inhibitors, including 7, 8-benzoflavone and 1-Maackiain acetate, to characterize the AHH activities of uninduced, and polycyclic hydrocarbon-induced rat tissues (liver, lung and kidney) and to characterize the AHH activities of microsomes from normal human liver and cigarette smokeinduced human placenta.

Methods Employed: Microsomal preparations are made 1) from, liver, lung and kidney of untreated rats and 9 rats treated with methylcholanthrene and phenobarbital, and 2) from human liver and human placenta. AHH activity is determined for each of these preparations with and without the addition of the inhibitors studied. Metabolic analysis of benzo(a)pyrene metabolism is performed by high pressure liquid chromatography.

Major Findings: 7, 8-benzoflavone and 1-Maackiain acetate are inhibitors that affect different forms of AHH. 7, 8-benzoflavone inhibits the AHH from methylcholanthrene-induced rat tissues, but has little or no inhibitary effect or stimulates the AHH from non-induced or phenobarbital-induced rat tissues. 1-Maackiain acetate greatly inhibits AHH from non-induced and phenobarbital-induced rat tissues, but has no inhibitory effect or stimulates the AHH from methylcholanthrene-induced rat tissues. This indicates the existance of at least two forms of AHH in rat liver, lung and kidney tissue. 7, 8-benzoflavone and 1-Maackiain acetate have similar effects on the AHH of the human tissues studied. 7, 8-benzoflavone stimulates the AHH of normal human liver tissue and strongly inhibits the AHH of cigarette smoke-induced placental tissue. In contrast, 1-Maackiain acetate strongly inhibits the AHH of normal human liver and has little effect on the cigarette smoke-induced AHH of human placenta.

Significance to Biomedical Research and the Program of the Institute: 1-Maackiain acetate and 7, 8-benzoflavone may be useful inhibitors of specific forms of AHH in humans as well as animal tissues. The unusual specificity of 1-Maackiain acetate as an inhibitor may make it a valuable tool for probing the enzymology and active catalytic sites of the different forms of cytochrome P-450 and for assessing the factors that determine the routes of PAH metabolism. This inhibitor may help in clarifying the relationship between drug and carcinogen activation and detoxification. It is possible that 1-Macckiain acetate and 7, 8-benzoflavone may be powerful natural inhibitors or modulators of chemical carcinogen-induced cancer.

Proposed Course: To examine the effects of specific inhibitors on tumorigenesis by various classes of carcinogens. To investigate other classes and related flavonoids as modulators of carcinogenesis

Publications:

Gelboin, H. V., West, D., Gozukara, E., Natori, S., Nagao, N., and Sugimura, T.: (-)Maackiain Acetate: A specific inhibitor or different form of aryl hydrocarbon hydrozylase in rat and human tissues. Nature (In Press)

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05208-01 LMC

PERIOD COVERED
September 1, 1980 to October 30, 1981

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies to Cytochrome P-450 and Characterization of Aryl Hydrocarbon Hydroxylase and 7-Ethoxycoumarin Deethylase of Human Tissues.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Tadahiko Fujino	Expert	LMC	NCI
Others:	Harry V. Gelboin	Chief	LMC	NCI
	Sang Shin Park	Visiting Associate	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Diversity of mixed-function oxidases in human placenta microsomes, monocytes and lymphocytes was investigated using monoclonal antibodies to 3-methylcholanthrene (MC)-induced rat liver cytochrome P-450. Monoclonal antibodies specific to MC-induced P-450 were obtained by hybridizing mouse myeloma cells with spleen cells of mice immunized with MC-induced rat liver cytochrome P-450.

Monoclonal antibodies to rat liver cytochrome P-450 not only inhibited the aryl hydrocarbon hydroxylase (AHH) of MC-induced rat liver microsomes but also inhibited AHH of human placenta and lymphocytes, 90% and 50%, respectively. The inhibitory effects of monoclonal antibodies of AHH of monocytes was negligible.

7-Ethoxycoumarin deethylase activity of human placenta and lymphocytes was inhibited 50%. No inhibition of these activities was observed with monocytes. The cross-reactivity of monoclonal antibodies to rat MC-induced cytochrome P-450 may be useful for studying the diversity and multiplicity of cytochromes P-450.

Project Description

Objectives: It is known that cytochrome P-450 from a single tissue has a range of substrate specificities. The multiple forms of microsomal cytochrome P-450 might account for the activation and detoxification of drugs, mutagens and chemical carcinogens. We are using P-450 specific monoclonal antibodies to investigate the diversities and multiplicity of P-450s. Benzo(a)pyrene, a ubiquitous chemical carcinogen in our environment, is metabolized into more than 40 metabolites through complicated metabolic pathways. We are using specific monoclonal antibodies to assess the involvement of specific species of P-450 in the metabolism of benzo(a)pyrene. The clarification of these metabolic pathways with specific monoclonal antibodies may lead the way to information which will be pertinant to the detection and prevention of cancer by chemical carcinogenesis.

Methods Employed: Monoclonal antibodies were obtained from hybridomas made by fusions of myeloma cells and spleen cells derived from BALB/c mice immunized with MC-induced rat liver cytochrome P-450. Human monocytes and lymphocytes were isolated from peripheral blood and treated with benzoanthracene. Human placental microsomes were prepared from placenta from women who smoke. AHH and 7-ethoxycoumarin deethylase activities were measured with a fluorospectrophotometric assay after incubation with monoclonal antibodies. The effects of monoclonal antibodies on the metabolism of benzo(a)pyrene by cytochrome P-450 were also studied by high pressure liquid chromatography.

Major Findings: 1) Monoclonal antibodies to MC-induced rat liver cytochrome P-450 not only inhibited the AHH activity of MC-induced rat liver microsomes but also inhibited the AHH activity of human placental microsomes. 2) The AHH activity of human placenta was inhibited 90% by monoclonal antibodies to MC-induced rat liver cytochrome P-450, while the 7-ethoxycoumarin deethylase was inhibited no more than 50%. 3) The AHH and 7-ethoxycoumarin deethylase activity of human lymphocytes pretreated with benzoanthracene were inhibited 50% by monoclonal antibodies, while these activities were not inhibited at all in benzoanthracene-treated monocytes.

Siginificance to Biochemical Research and the Program of the Institute: The monoclonal antibody approach provides us with the ability to analyze the complicated phenomenon of polycyclic hydrocarbon metabolism from a new perspective. Use of monoclonal antibodies to cytochrome P-450 should clarify the metabolic pathways responsible for chemical carcinogenesis. Knowing the enzymatic steps leading to the activation of carcinogen will be useful for the detection and prevention of chemical carcinogenesis.

Proposed Course: Monoclonal antibodies to cytochrome P-450 will be further explored. These might attack different antigenic determinants of cytochrome P-450 which have not yet been clarified by ordinary methods. The diversity of enzymes will be studied further with these specific monoclonal antibodies. The diversity and multiplicity of cytochrome P-450s and their catalytic specificities will be studied further using monoclonal antibodies and HPLC analysis of polycyclic hydrocarbon metabolism. Monoclonal antibodies which should be specific for other antigenic sites on the cytochrome P-450 molecule are now in preparation. These, as well as the monoclonal antibodies now in hand, will be used in these studies.

Publications:

None

THE LABORATORY OF MOLECULAR VIROLOGY
October 1, 1980 through September 30, 1981

The Laboratory of Molecular Virology (1) analyzes the mechanism of gene expression in normal and transformed eukaryotic cells; (2) plans and conducts research on viruses to define their potential role in the development of cancers in animals and man; (3) develops and applies biological, biochemical and immunological procedures to obtain evidence for the presence of virus genetic information in cells; (4) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells; (5) and uses viruses as tools for probing cellular regulatory mechanisms.

The Microbiology Section (1) applies virologic, biochemical, and immunologic techniques to the study of the biology of herpesviruses and the nature of their association with certain cancers in humans and animals with emphasis on the elucidation of the mechanisms whereby viruses of this group establish occult infections in cells which terminate in neoplasia; and (2) defines the role of herpesviruses in oncogenesis by investigations of their effect upon normal cellular control mechanisms and the effects of herpesvirus and Type C RNA virus interactions in dually-infected cells. The Virus Tumor Biology Section (1) characterizes the nucleotide sequence from regions of viral and cellular DNA thought to be involved in gene expression; (2) investigates the properties of transforming genes from viruses, and their protein products; evaluates the effects of viral infection on cellular control mechanisms by comparing transcription and translation of virus genome information in transformation and in lytic responses to infection; and (3) compares isolated transforming genes to homologous genes isolated from normal cells and identifies molecular elements responsible for transforming activities. The Virus Tumor Biochemistry Section (1) investigates the molecular elements essential for cellular transformation. Specific regions of viral and cellular DNA thought to be involved in the expression of cellular transforming phenotopes are isolated by recombinant DNA techniques. Structural properties of molecularly cloned fragments are determined and their transforming activities characterized in biological assays; and 2) has expertise in prokaryotic and eukaryotic molecular biology and uses recombinant DNA techniques and molecular genetics to study the effect of genomic recombination on the expression of isolated viral and cellular genes. Specific eukaryotic genes are recombined in prokaryotic host-vector-systems and their expression and mode of chromosomal association is examined in eukaryotic hosts. The Cell Physiology Section is vacant at the present time.

A major objective of the Laboratory of Molecular Virology remains the elucidation of the regulatory signals which are responsible for gene expression. In this regard, we have defined an important regulatory element in the expression of the early SV40 mRNAs, specifically the 72 base pair repeated segments to the late side of the replication origin. This particularly interesting segment of the genome is unique in that it encodes no known polypeptides, and appears to be free from nucleosomes as judged by sensitivity to nuclease digestion in SV40 chromatin. A number of studies are in progress to define the specific nucleotides within this segment which are critical to transcriptional initiation, as well as to determine the role of similar sets of nucleotides in other viral and cellular operons. The role of splicing in mRNA stability has been pursued in detail. A mutant has been constructed from an SV40 rat preproinsulin recombinant molecule which generates abundant quantities of stable late unspliced mRNAs. We are continuing experiments designed to determine which mRNAs do or do not require splicing as a mechanism for enhancing the efficiency of transcription and/or export of mRNAs to cytoplasm.

Considerable effort has been directed at the development of both prokaryotic and eukaryotic vectors which will lead to the efficient expression of a set of well-defined genes. In terms of prokaryotic vectors, we have concentrated on enhancing efficiency by synthetically constructing what may be considered to be an optimal ribosome binding site. This is based on previous observations which lead to the conclusion that ribosome binding plays a critical role in the efficiency of translation of prokaryotic messages. Future studies will be directed toward further enhancement of these prokaryotic vectors through optimization of transcriptional promoters. A number of studies have been performed using SV40 as a eukaryotic vector. Most notably, we have obtained the efficient expression of the rat proinsulin polypeptide and the Harvey sarcoma virus transforming protein p21, in monkey kidney cells infected by SV40 recombinant molecules in the presence of helper viruses. In subsequent collaborative efforts, we have contributed to developing the bovine papilloma virus vector system which appears to offer the advantage of continuous expression of the gene of interest in a permanently transformed cell. Furthermore, BPV recombinant molecules seem to reside entirely in an unintegrated form which should allow for easy recovery of the molecules of interest.

A number of studies were directed at further understanding of the proteins involved in SV40 transformation events. We were able to show that the SV40 and adenovirus transforming proteins share a number of properties, and in fact, are able to substitute for one another in several functional analyses. It was also demonstrated that a small fraction of the SV40 T-antigen population contains a novel modification, poly-ADP-ribosylation. Future studies are directed at determining the significance of this modification and whether or not it correlates with the particular subset of T-antigens which may perform a critical role in either DNA replication, transcriptional regulation, or cell transformation. Another approach to developing a functional assay for T-antigen involved the establishment of an SV40 DNA replication system. Using this system, it was shown that wild type T-antigen plays an essential role in the *in vitro* replication of SV40 DNA molecules; the T-antigen derived from a transformed line of human cells (SV80) was found to be defective in the replication function as judged by its inability to substitute in these *in vitro* assays. A further finding was the presence of a putative cellular protein present only in rapidly growing (log phase) cells and absent from stationary cells which can subsitute for the SV40 T-antigen in promoting viral DNA replication. As might be expected, however, this cellular polypeptide is much less efficient than the viral protein itself. Future studies will be directed at the elucidation of the cellular proteins which bear resemblance in this *in vitro* assay to the SV40 T-antigen.

A number of groups, including our own, have shown that a cellular protein is routinely present in tumor cells transformed by various agents including X-rays, chemicals, and DNA or RNA tumor viruses. In the mouse, this polypeptide has a molecular weight of 53,000; similar related proteins, which vary slightly in size, have been found in transformed cells from a variety of other species including rat, hamster, rabbits and human cells. We have shown that the mouse p53 is present in certain rapidly growing human cells, such as early passages of kidney epithelium, and thymus cells. The p53 polypeptides are immunogenic in their species of origin; however, various tumors differ in their capacity to induce p53 antibodies. This polypeptide has been shown to be phosphoprotein which has an associated protein kinase activity and can be induced in normal spleen cells by treatment with lectins.

A novel SV40 encoded polypeptide, the so-called agnoprotein, has for the first time been isolated and defined. This protein arises during the late phase of the lytic cycle, has a rapid turnover rate, can efficiently bind to single- or double-stranded nucleic acids, and appears to be absent from transformed cells. In future studies, agnoprotein-specific antiserum will be derived which should help to elucidate cellular localization of this polypeptide, as well as its potential function.

A number of studies have been directed at an understanding of the molecular organization and expression of cell surface antigens. These include the mouse H-2 and Lyt species. In the case of the former, cDNA and genomic clones have been obtained which are presently being analyzed at a structural level. Future studies will be directed at determining the level of expression of these polypeptides, as well as the DNA and RNA sequences which encode them in specific tissues. The Lyt antibodies were analyzed by immunoprecipitation and subsequent polyacrylamide gel electrophoresis. A particularly interesting finding was the presence of 3 subunits of 35,000, 30,000, and 28,000 daltons. Further experiments indicated that two forms of a particular Lyt antigen consisted of a 28,000 molecular weight subunit covalently associated through a disulfide linkage with either a single 35,000 or a single 30,000 M_r subunit.

The major research endeavors of the Biochemistry Section are to identify and characterize the molecular elements from viral and cellular genomic DNA responsible for transformation. During the past year, we have made considerable progress in identifying such elements in acute transforming retroviruses and in normal cellular genomic DNA. We have previously described the structure and biological activity of cloned Moloney sarcoma (MSV) proviruses. These studies showed that a cloned internal subgenomic MSV fragment, containing the acquired cell sequence v-mos, was able to transform cells at low efficiency in DNA transfection assays. When v-mos was covalently linked to MSV sequences containing the long terminal repeat (LTR), the transforming efficiency was enhanced one thousand-fold. These studies were first to demonstrate biological enhancement of transformation by a retrovirus provirus LTR element placed either upstream or downstream to a transforming sequence.

We identified and isolated a normal mouse cell fragment containing sequences homologous to v-mos, namely c-mos. This c-mos containing fragment was unable to transform cells. We asked whether the addition of an LTR element to c-mos would activate its ability to transform cells in a manner analogous to its ability to enhance v-mos transformation. A series of recombinants was constructed with the LTR placed at variable distances (from 500-1500 base pairs) upstream from c-mos. All recombinants transformed cells efficiently. Activating c-mos transforming potential by an LTR is analogous to a retrovirus integrating in the host chromosome upstream from a sequence with transforming potential. In fact, investigators at Rockefeller University have identified a similar LTR-transforming sequence configuration in the DNA from tumors induced by avian leukosis virus. In 30 out of 36 lymphomas analyzed, the avian retrovirus LTR and, presumably the provirus, were found integrated upstream from a normal cell sequence refered to as c-*myc*. They have not yet determined whether LTR - c-*myc* DNA fragments transform cells like the LTR - c-*mos* recombinants, but they have detected RNA transcripts in these tumors containing LTR and c-*myc* sequences. In our studies, cells transformed by v-*mos* or c-*mos*, with LTR elements positioned either upstream or downstream to the transforming sequence, express RNA in the same configuration. Thus, the LTR element appears to activate RNA expression of the *mos* sequence when it is positioned either before or after this sequence. This latter configuration has also been detected by Bishop and his colleagues in avian leukosis virus-induced lymphomas, i. e., c-*myc* - LTR. Thus, the LTR appears to have transcription activation properties in addition to providing signals for either starting or polyadenylation of RNA transcripts. Movement or activation of sequences with LTR-like properties adjacent to sequences with transforming potential may be a general mechanism for oncogenesis.

In a model for transformation whereby a retrovirus integrates adjacent to a normal cell gene with transforming potential, it is important to know whether the methylated state of this DNA influences LTR activation of transformation. We have found that methylation of v-*mos* results in a reduction in transforming efficiency. A similar reduction is observed when the LTR is methylated. When both v-*mos* and LTR sequences are methylated, the reduction in transforming frequency is equivalent to the reduction observed when the entire MSV provirus is methylated. These studies suggest that the methylated state of a cell sequence with transforming potential will influence its activation by integrated provirus LTR elements.

In addition to providing us with the first insight as to how a normal cell sequence with transforming potential may be activated to transform cells, studies with MSV and v-*mos* have indicated how the provirus can be used as a cloning vehicle. We have used a portion of the MSV provirus, consisting of the MSV LTR and *gag* region, as a vector. As with the MSV LTR, we have been able to enhance the transforming efficiency of v-*mos* with this vector. More importantly, we have been able to rescue defective transforming viruses from cells transformed by v-*mos* and this vector by infecting them with a competent helper retrovirus. Understanding this process can provide a powerful tool for identifying and isolating other cellular sequences with transforming potential.

We have continued our analyses of the novel rearrangements found in the defective DNA of herpes simplex virus type 1 (HSV-1) and have identified three structural groups. All of the structural groups possess sequences derived from the terminal and internal repeated sequence (TRS/IRS) of the S region. In structural group one, additional sequences are derived from the contiguous, unique S region (in the prototype orientation of the genome). In group two, the additional sequences are derived also from contiguous unique S region sequences, but from the opposite permutation. In group three, the most unusual set of defective DNA molecules, sequences are derived from non-contiguous portions of both the unique S and L regions. Models have been proposed to describe the mechanism whereby group one, group two, and most of group three structures could be derived from the wild type parental genome. Examination of the genomic DNA from a number of single plaque isolates of wild type HSV-1 has shown hyper-variability in the site where structural group one defective DNA molecules are generated. Analysis of this region may provide insight into the mechanisms for defective DNA generation. We expect that analyses of these novel rearrangements will reveal mechanisms for eukaryotic recombination.

High frequency deletions have been shown to occur in lambda recombinants containing the entire unique S region and portions of the TRS/IRS. A large number of such deletions have been isolated and have been used to generate a Sma I map of this region.

We have continued analyses of the regulatory and transcriptional elements possessed by sequences in defective DNA. The TRS/IRS sequence possesses promoter signals for two immediate early messenger RNA's (mRNA's). For one of these RNA's, the entire structural gene is encoded within the TRS/IRS and, therefore, occurs twice within HSV-1 genome. The second RNA promoted by this region has been shown to possess an intron encoded within the TRS/IRS, but sequences encoding putitive polypeptides are encoded within the unique S region. The complete nucleotide sequence of one of these polypeptides has been determined. These studies represent the first comprehensive analyses of an immediate early HSV-1 mRNA.

A host range mutant of HSV-1 was developed whose replication was restricted in rodent, but not primate cells. The mutant without prior inactivation could tumorigenically transform mouse cells and could enhance the transformation of mouse cells by SV40. A non-transformed thymidine kinase minus (tk-) mouse cell was developed. Introduction of the HSV-1 tk gene either by infection with uv-irradiated HSV-1 or by transfection with the cloned tk gene did not result in morphological transformation of the tk converted cells. A battery of monoclonal antibodies to HSV-1 proteins were prepared and are being utilized for developing serological tests and for identifying any HSV products being expressed in transformed cells.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04876-09 LMV

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Oncogenic Virus Influence on the Biochemical Events of Host Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul S. Ebert	Research Chemist	LMV, NCI
OTHER:	G. F. Vande Woude	Chief, VTBS	LMV, NCI

COOPERATING UNITS (if any)

D.P. Tschudy	C, MET	P.D. Smith	H, IR, TD
E.C. Weinbach	NIAID, LPP	R.F. Bonner	R, BEI
J.L. Costa	M, CN		

LAB/BRANCH

Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION

Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Succinylacetone (SA), a potent specific inhibitor of heme biosynthesis, was shown to bind irreversibly to δ-aminolevulinic acid dehydrase, the second enzyme in the heme biosynthetic pathway. SA inhibits the growth of Walker carcinoma (W256) in vitro and in vivo, the Novikoff hepatoma in vivo, and L1210 leukemia in vitro, but only slightly in vivo. Since cellular heme levels are not decreased in W256 and L1210 cells, another unkown mechanism of growth inhibition is functioning. The characteristics of heme uptake were examined in 3 malignant cell lines, a normal rat liver cell line, and a normal embryonic cell. The normal liver cell line, BRL-3A took up hematin at a slow rate while hematin uptake in murine erythroleukemia (MEL) cells, W256, L1210, and chick embryo fibroblasts was more rapid and of greater magnitude. Saturation of the heme uptake mechanism occurred at a much higher cellular heme concentration in L1210 cells than in MEL cells. Hematin uptake in MEL cells could be markedly stimulated by pretreatment of the cells with DMSO, procaine, detergent, or proteolytic enzymes or by increases in the pH of the medium from 8 to 9.5. SA caused a gradual increase in hematin uptake capacity in MEL cells over a period of days.

PROJECT DESCRIPTION

Objectives:

To determine the mechanism of antitumor action of succinylacetone in non-hematopoietic cancer cells. To study the mechanism of action of the selective uptake of porphyrins in malignant cells and ways to amplify this effect to achieve greater differential heme concentrations between tumor and adjacent normal tissues. To determine why malignant cells have a high requirement for heme.

Methods Employed:

Conventional biochemical and tissue culture procedures were utilized to follow alterations in heme synthesis and growth of tumor cells. Live cells were determined by trypan blue exclusion. Hematin and hematoporphyrin were determined by boiling the samples in oxalic acid and reading the resultant fluorescense in a fluorimeter.

Major Findings:

1. Uptake of hematin in malignant, embryonic, and normal cells. The characteristics of hematin uptake were examined in three malignant cell lines: (1) L1210 leukemia, (2) 745 murine erythroleukemia (MEL), and (3)Walker carcinoma (W256), a cell line derived from normal rat liver (BRL-3A), and a normal embryonic cell, chick embryo fibroblasts (CEF). Uptake of hematin in the normal BRL-3A line was slight and occured at a slow rate. In contrast, the three malignant cell lines showed rapid hematin uptake which was nearly maximal within 2.5 minutes after exposure to hematin. The embryonic line also took up hematin rapidly within two minutes, but additional uptake continued for thirty minutes. Saturation of the heme uptake mechanism was observed in MEL cells at an extracellular hematin concentration of 160 uM, while the saturation level in the much more malignant cell line, L1210 was at almost two times that concentration. At saturation L1210 cells achieved a cellular heme concentration 9 times as high as MEL cells. Small differences in hematin uptake at 4°, 20°, and 37° in MEL and W256 cells suggest that no enzymatic process is involved. Hematin uptake was markedly augmented in MEL cells by increasing the pH of the medium from 8 to 9.5. Increases in pH in this range of values did not augment hematin uptake in L1210 or W256 malignant cells, or the normal liver or embryonic cells. After exposure of MEL cells to a high concentration of hematin in the medium, the loss of heme was followed under various conditions. Of the various agents studied, only cyanide produced a loss of heme from MEL cells.

2. Effect of succinylacetone (SA) and membrane-active agents on hematin uptake.

Hematin uptake in MEL cells was markedly augmented by short-term pretreatment with DMSO, procaine, detergent, or 2 proteolytic enzymes. In L1210 cells pretreatment with trypsin, DMSO, and procaine also stimulated the uptake of hematin above that seen in cells treated with hematin only. Trypsin treatment of BRL-3A cells did not increase the amount of hematin uptake above that of cells receiving hematin alone. In chick embryo fibroblasts pretreatment with trypsin and DMSO, as with the malignant cells, stimulated an increase in hematin uptake. In contrast to the observation that DMSO and procaine stimulate hematin uptake after a short term incubation, SA caused a gradual increase in hematin uptake capacity in MEL cells over a period of 2-5 days, suggesting the development of an induction mechanism.

3. Effect of SA on the growth of W256 and L1210 cells in vitro and in vivo. Agents, such as methotrexate, might be attached to the porphyrin molecule to facilitate their selective passage into tumors and cancer cells. The use of hematoporphyrin derivative in photoradiation therapy in human carcinoma has already been demonstrated.

Significance to Biomedical Research and the Program of the Institute:

Succinylacetone, a potent inhibitor of the heme pathway, has shown antitumor activity against L1210 leukemia and W256 carcinoma in vitro and against W256 and Novikoff hepatoma in vivo in rats. Its high water solubility and its apparent low toxicity in animals and the fact that the compound was originally isolated from human tissue are characteristics which recommend it for further in vitro and animal testing. The compound will be very useful for biochemical studies involving inhibition of heme synthesis, protein synthesis, and respiration. Since hematin and hematoporphyrin are selectively taken up by tumor cells, several medical uses can be anticipated. Labeled with isotopes these porphyrins can serve as scanning agents and tumor localizers. Certain potent chemotherapeutic agents, such as methotrexate, might be attached to the porphyrin molecule to facilitate their selective passage into tumors and cancer cells. The use of hematoporphyrin derivative in photoradiation therapy in human carcinoma has already been demonstrated.

Proposed Course:

Further in vivo experiments will be run to enhance the antitumor activity of SA against L1210 leukemia in mice. We will further characterize the antitumor activity of SA against L1210 leukemia, since it appears to kill these tumor cells by some unknown mechanism not related to the inhibition of heme biosynthesis. We will determine if the augmentation of hematin uptake by several membrane-active agents observed in vitro also occurs in animals bearing ascites tumors. Photoradiation therapy has been successfully utilized against human tumors which had been previously exposed to hematoporphyrin, but the methodology has not been maximized. We will explore several of the variables involved in photoradiation therapy with L1210 cells in vitro and apply the information gained to magnify the antitumor response in rodents. Visible light of five different wavelengths as well as pulsed laser light, will be evaluated.

Publications:

Tschudy, D.P., Ebert, P.S., Hess, R.A., Frykholm, B.C. and Weinbach, E.C.: The effect of heme depletion on growth, protein synthesis, and respiration of murine erythroleukemia cells. Biochem. Pharm., 29: 1825-1831, 1980.

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Ebert, P.S., Bonkowsky, H. L. and Wars, I.: Stimulation of hemoglobin synthesis in murine erythroleukemia cells by low molecular weight ketones, aldehydes, acids, alcohols and ethers. Chemico-Biol. Interactions, 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04882-07 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Events in Tumor Virus Replication and Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George F. Vande Woude	Chief, VTBS	LMV, NCI
OTHER:	R. Ascione	Research Chemist	LMV, NCI
	L.W. Enquist	Research Scientist, PHS	LMV, NCI
	W.L. McClements	Senior Staff Fellow	LMV, NCI
	T. G. Wood	Senior Staff Fellow	LMV, NCI
	R. Watson	Fogarty Fellow	LMV, NCI
	K.D.-Thompson	Senior Staff Fellow	LMV, NCI
	S. Bear	Staff Fellow	LMV, NCI
	M.L. McGeady	NIH Fellow	LMV, NCI
	P.J. Fischinger	Chief, TVS	LVC, NCI
	D.G. Blair	Expert	LVC, NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Cloned Moloney murine sarcoma virus (MSV) proviral DNA, containing the acquired cell sequence v-mos and the long terminal repeat (LTR) provirus transcription control element, transforms cells efficiently in DNA transfection assays. In contrast, a normal mouse cell DNA sequence (c-mos) homologous to the transforming sequence of MSV was shown to be inactive in this assay. The transforming potential of c-mos was activated by forming recombinant molecules containing the LTR positioned from 500-1500 base-pairs upstream from the 5' end of the cellular mos. These results suggest a model for oncogenesis whereby a normal cell gene with transforming potential is activated by a transcription control element to express its transforming phenotype.

A normal human cell homolog of mos was cloned and compared to the MSV and mouse v-mos sequence. Heteroduplex analyses revealed that under non-stringent conditions the human mos was co-linear with the mouse mos. However, most of the six base restriction enzyme recognition sites of v-mos are not conserved in the human homolog.

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2) Methylation of MSV proviral DNA by Hpa II methylase reduces by ten-fold the transforming efficiency of this DNA in a DNA transfection assay. By specifically methylating each of the two regions of the provirus required for efficient transformation (the LTR and *mos* regions) and co-transfecting the DNAs, it was found that methylation of LTR alone decreased focus-formation three-fold and of the *mos* region alone, ten-fold. When both regions were methylated, a thirty-fold decrease in transformation efficiency was observed.

3) Detailed RNA and sequence analyses have identified the sequences in HSV-1 DNA coding for the 5' leader and introns of two immediate early mRNAs (IE mRNA-4 and -5). These sequences occur within the terminal inverted repeat of the HSV-1 *S* region. The reading frames for both of these IE mRNAs begin within the unique *S* region. The complete sequence of the IE mRNA-5 is predicted from sequence analyses.

PROJECT DESCRIPTION

Objectives:

To investigate biochemical events associated with tumor virus replication and to determine their relationship to neoplastic transformation of host cells.

Methods Employed:

Oncogenic viruses are propagated in tissue culture. Focus assays and plaque assays are used for the detection of some propagated and purified viruses. Other assays are: agarose gel diffusion for the detection of viral antigens, polyacrylamide gel electrophoresis and guanidine-agarose chromatography for the purification and identification of virus-specific proteins, *in vivo* and *in vitro* labeling of purified viruses using radio-labeled amino acids and nucleic acids. Sensitive peptide mapping techniques are developed for use in comparing virion polypeptides. Endonuclease restriction enzyme cleavage of viral and cellular DNA. Cloning in bacterial and mammalian vectors of specific virus-related sequences of viral and host cell origin.

Major Findings:

I. We previously demonstrated that in a DNA transfection assay MSV proviral DNA transforms cells in culture efficiently. The regions responsible for transformation were found to be the v-*mos* region, a normal cellular DNA sequence acquired by the virus, and the long terminal repeat (LTR), a transcriptional control region. While the v-*mos* region transformed cells at low efficiency, the efficiency of transformation by v-*mos* could be increased one thousand-fold by addition of an LTR element. We asked whether the normal cell homolog of v-*mos* (c-*mos*) would transform cells in culture. Cloned mouse DNA fragments containing c-*mos* were found to be completely inactive in the transformation assay. When a c-*mos*-containing fragment was linked to a 5' fragment of MSV proviral DNA containing an LTR element and leukemia virus sequences, the hybrid

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clone transformed efficiently. To determine whether LTR alone could activate the c-*mos* sequence, a series of hybrid clones containing LTR positioned from 500-1500 base-pairs upstream from c-*mos* were constructed. All of these recombinants transformed cells efficiently indicating that LTR provided the necessary transcriptional control elements for expression of c-*mos*. This suggests a molecular model for oncogenesis whereby any genomic rearrangement which places or activates a transcriptional control element adjacent to a sequence with transforming potential can result in the expression of the transformed phenotype.

Southern analysis of human placental DNA digested with *Bam* H1 and *Eco* R1 endonuclease restriction enzymes reveal a single hybridizing DNA band with a mouse *mos* probe. Human c-*mos* homologs to MSV v-*mos* were cloned as *Eco* R1 and *Bam* H1 fragments. The v-*mos* and human *mos* regions were compared by heteroduplex analyses at 25° and found to be co-linear. However, while six base restriction enzyme recognition sites between the MSV v-*mos* and mouse c-*mos* are highly conserved, few, if any, of these sites have been conserved between the mouse and human c-*mos*.

II. We have asked what effect methylation of DNA exerts on transformation by MSV. Methylating intact MSV proviral DNA with Hpa II methylase decreased its transforming efficiency ten-fold. We had previously shown that two regions of the provirus were necessary for efficient transformation: the long terminal repeat (LTR), a putative transcriptional control element, and v-*mos*, the transforming sequence acquired by the virus from the normal cell genome. Co-transfection of a mixture of LTR and v-*mos* sequences also efficiently transforms cells. When LTR alone was methylated, a three-fold reduction in focus-formation was observed. When the *mos* region alone was methylated the reduction was ten-fold. When both DNA's were methylated the transforming efficiency was reduced thirty-fold. These results indicate that methylation of a sequence with transforming potential could be an inhibiting or controlling factor which prevents its activation by a transcriptional control element. Thus, activation of cell genes by upstream integration of a retrovirus could be influenced by the state of methylation.

III. Research into the detailed structures of the genes specifying two herpes simplex virus type 1 (HSV-1) immediate-early mRNAs (IE mRNA-4 and -5) has been continued. The virus immediate-early RNAs are transcribed by pre-existing cellular polypeptides in cells infected with HSV-1 in the continuous presence of protein synthesis inhibitors. IE mRNA-4 and IE mRNA-5 are spliced molecules which map at the two junctions of the unique (U_S) and inverted repeated regions (TR_S/IR_S) of the virus DNA short component. We have shown, previously, that IE mRNA-4 and IE mRNA-5 contain a similar 260 base (b) 5' terminal co-transcript mapping within TR_S/IR_S. This leader sequence is spliced to 3' terminal co-transcripts of 1450 b (for IE mRNA-4) and 1540 b (for IE mRNA-5) containing predominantly U_S sequences, but with a small amount of homology to TR_S/IR_S. The gene introns (estimated to be 150 b) are, therefore, located entirely with the inverted repeats.

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We have subsequently determined the complete DNA sequence of the IE mRNA-5 gene and a partial sequence of the IE mRNA-4 gene. Using a nuclease S1 analysis, the 5' terminal nucleotides of IE mRNA-4 and IE mRNA-5, and the 3' terminal nucleotide of IE mRNA-5, have been determined. Further, the exact location of the IE mRNA-5 gene intron has been found by sequencing cDNA synthesized using a primer specific for IE mRNA-5. Therefore, the entire sequence of IE mRNA-5 can now be predicted.

These sequence analyses have allowed the following conclusions to be drawn: (1) the exons encoding the 5' leader sequences of IE mRNA-4 and -5 have identical sequences and are 258 or 260b in length (the transcripts initiating at an A or a G); (2) the gene introns are identical, are 149b in length and consist predominantly of a 23b sequence tandemly repeated four times, plus a further 15b partial repeat; (3) the 3' co-transcripts contain 64b of TR_S/IR_S sequence; (4) the first putative initiator codon (ATG) maps 6 bases into the U_S sequences specifying IE mRNA-5, from which the reading frame is open for 87 codons (enough to code for a polypeptide of ~10,000 MW).

The sequences bounding the DNA encoding IE mRNA-5 share certain common characteristics with those of other genes transcribed by RNA polymerase II. Experiments are now in progress to determine which 5' IE mRNA-5 sequences are involved in the regulation of transcription. These studies involve linking *in vitro* the 5' sequences of the IE mRNA-5 gene to the coding sequence of the HSV-I thymidine kinase gene. The strength of the promoter, both *in vitro* or *in vivo*, may then be measured by assaying for thymidine kinase activity. The effects of modifications of the promoter region can then be assessed.

Significance to Biomedical Research and the Program of the Institute:

The formation of hybrid molecules between transcription control elements and cellular genes with transforming potential can serve as a model for identifying the molecular elements essential for transformation. Results suggest a model whereby retroviral integration at multiple sites in the host chromosome could serve to activate expression of normally quiescent cellular sequences with transforming potential mediated through an LTR-like transcription control element. This may be a useful model for other, non-viral, transformation events. Thus, normal cell sequences with functional properties of an LTR (e.g. transposons) could be transposed adjacent to transforming sequences or activated to express adjacent transforming sequences as a result of a genomic insult.

Proposed Course:

1. Specific cellular *onc* genes from human genomic DNA will be isolated, characterized and tested for their ability to transform cells.

2. The early events in herpes simplex virus replication will be characterized at the molecular level.

Publications:

Dhar, R., McClements, W.L., Enquist, L.W. and Vande Woude, G.F. Nucleotide sequence of integrated Moloney sarcoma virus long terminal repeats and their host and viral junctions. Proc. Natl. Acad. Sci. USA 77: 3937-3941, 1980.

Blair, D.G., McClements, W.L., Oskarsson, M. and Vande Woude, G.F. The long terminal repeat of Moloney sarcoma provirus enhances transformation. Haem. and Blood Trans. 26: 460-466, 1981.

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McClements, W.L. and Vande Woude, G.F. Cloning retroviruses: Retrovirus cloning? Genetic Engineering, Vol. III, Plenum Press, pp. 699-705, 1981.

Blair, D.G., Oskarsson, M.K., Wood, T.G., McClements, W.L., Fischinger, P.J. and Vande Woude, G.F. Activation of the transforming potential of a normal cell sequence: A molecular model for oncogenesis. Science 212: 941-943, 1981.

Watson, R.J., Sullivan, M. and Vande Woude, G.F. Structures of two spliced herpes simplex type 1 immediate early mRNA's. J. Virol. 37: 431-444, 1981.

Denniston, K.J., Madden, M.J., Enquist, L.W. and Vande Woude, G.F. Characterization of coliphage lambda hybrids carrying DNA fragments from herpes simplex virus Type 1 defective interfering particles, Gene, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04902-08 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

In Vivo and In Vitro Translation of Tumor Virus Specific Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Ascione	Research Chemist	LMV, NCI
OTHER:	G. F. Vande Woude	Chief, VTBS	LMV, NCI
	Roger Watson	Fogarty Fellow	LMV, NCI

COOPERATING UNITS (if any)

Martin Zweig, Litton Bionetics

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have translated, in a cell-free system several immediate early (IE) herpes simplex virus type 1 (HSV-1) specific polypeptides using mRNA obtained from cycloheximide (CX)-treated HSV-infected cells. Specifically, we have identified the major IE polypeptide of 175,000 dalton synthesized in a messenger-dependent reticulocyte lysate (MDL) cell-free system. Product identification was confirmed by using monoclonal antiserum directed against HSV-1 infected cell products. We have also isolated late HSV-1 mRNA from infected cells and translated the RNA using a cell-free (MDL) system; several HSV-1 specific products have been identified using monoclonal antibody. Specifically, the interrelated 80,000 and 40,000 dalton proteins, as well as a 52,000 dalton polypeptide, have been identified by monoclonal antibodies. The latter protein was immunoprecipitated by the antiserum directed against the HSV-1 major glycosylated capsid protein gpD.

PROJECT DESCRIPTION

Objectives:

To determine the specific translation products of mRNAs and their genomic origins of herpes simplex virus type 1 (HSV-1) using nitro-cellulose selective techniques with cloned segments of HSV-1 DNA.

Methods Employed:

Guanidine extraction of whole cellular RNA from infected cells. Fractionation of RNA by oligo dT column chromatography. Preparation of rabbit reticulocytes for mRNA dependent cell-free protein synthesizing system. Cell-free translation by lysates using specific mRNA and analysis of synthesized polypeptides by linear-gradient-polyacrylamide gel electrophoresis (PAGE) and two dimensional analytical electrophoresis employing isoelectric focusing in the first dimension and PAGE analysis in the second dimension. Formation of specific hybrids with isolated mRNA using specific restriction fragments of HSV-1 obtained from cloned segments of viral DNA. Selection of infected cell mRNA by affinity column chromatography, specific binding and elution of HSV-1 mRNA using amino benzoxy methylcellulose (ABM-cellulose) covalently bound restriction fragments of HSV-1 DNA. Characterization of HSV-1 polypeptides by hybridoma derived monoclonal antibodies specific for unique viral products.

Major Findings:

A. Immediate early mRNA isolated from defective (d) and nondefective (nd) HSV-1 infected cells stimulates the synthesis of high molecular weight polypeptides. The predominant proteins synthesized in a cell-free translation system were 175,000, 135,000, 110,000 and 68,000 daltons in molecular weight. No major qualitative differences were noted between products specified by mRNA isolated from cells infected with nd or d HSV. However, there were quantitative differences in the amounts of 175,000 and 110,000 dalton products. Specifically, these polypeptides were 2-3 fold more abundant using the d-HSV-1 specific mRNA, while the 135,000 dalton polypeptide was reduced when compared to translation products of the nd-HSV-1 specific mRNA.

B. HSV-1 specific proteins synthesized in vivo, in the presence of cycloheximide, preferentially bind to DNA-cellulose. Similarly, 75% of HSV-1 IE mRNA polypeptides translated cell-free in MDL, bound to calf thymus DNA-cellulose. As much as 40% of the late HSV mRNA translated products bound to DNA-agarose. These cell-free synthesized products were similar to HSV-1 nucleocapsid proteins.

C. Monoclonal antisera prepared against the major HSV-1 glycoprotein, gpD, appears to have reactivity to a cell-free synthesized 52,000 dalton product. Since the MDL-synthesized counterpart is not glycosylated in vitro, a variety of monoclonal antisera will be screened to isolate one which is most efficacious in recognizing nonglycosylated sequences.

D. Monoclonal antisera prepared against an HSV-1 polyprotein of 80,000 daltons, sharing common antigenic determinants and tryptic peptides (M. Zweig, personal communication) with a 40,000 dalton polypeptide, precipitated polypeptides synthesized *in vitro*.

Significance of Biomedical Research and the Program of the Institute:

These studies serve to map the genomic location of herpesvirus polypeptides and their corresponding mRNA. Virion-associated DNA binding proteins can serve to characterize the possible regulatory function(s) associated with viral replication.

Proposed Course:

HSV-1 specific early and late mRNA will be isolated using cloned HSV-1 DNA fragments coupled to ABM cellulose. This mRNA will be translated to determine the protein(s) encoded in the specific fragment. Additionally, using specific monoclonal antiserum prepared against HSV-1 proteins will enable us to identify coding regions for structural, replication and transforming regions of HSV-1 DNA.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT**	**PROJECT NUMBER** Z01 CP 04914-04 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Integration, Excision and Rearrangement of Genetic Information

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lynn Enquist	Research Scientist (PHS)	LMV NCI
OTHER:	K. Umene	Fogarty Fellow	LMV NCI
	G. Vande Woude	Chief, VTBS	LMV NCI
	A. M. Colberg-Poley	Postdoctoral Fellow	LMV NCI

COOPERATING UNITS (if any)

R. Weisberg, LMG, NICHD, NIH
D. Nebert, DP, NICHD, NIH
M. Pearson, CBP, FCRC
T. Silhavy, CBP, FCRC

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda 20205

TOTAL MANYEARS:	**PROFESSIONAL:**	**OTHER:**
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our principal effort is directed to understanding the mechanisms by which novel DNA rearrangements occur in vivo. We have concentrated on two major systems: bacterial viruses capable of integration and excision and herpes simplex virus (HSV). Phage lambda is used for both in vivo and in vitro recombination systems and for cloning DNA fragments of HSV. We are analyzing the mechanism by which phage lambda integrates through genetic manipulation of the integrase enzyme as well as through isolation of phages with altered integration sites. We are using lambda-HSV recombinants to define and characterize regions of HSV DNA rearrangement. We are analyzing one HSV fragment that is very unstable when propagated in E. coli. A fine structure deletion map of the HSV Us region has been constructed using lambda-HSV hybrids. We have initiated a collaboration on the gene structure and rearrangement of the mouse cytochrome p450 complex and have cloned a cDNA that represents part of the p450 gene induced by the carcinogen methylcolanthrene.

PROJECT DESCRIPTION

Objectives:

To understand how a virus inserts its genome into the host genome; to understand how specific DNA sequences are recognized by insertion-excision enzymes; to study regulation of virus encoded recombination functions; to isolate sites on HSV DNA involved in genomic rearrangement; to utilize prokaryotic technology to analyze eukaryotic genomes; and to understand genetic rearrangement as it relates to gene expression.

Major Findings:

I. Expression of the int gene after phage λ infection normally requires the products of genes cII and cIII. However, when the deletion in the nonessential b2 region is adjacent to int, efficient synthesis of active int protein does not require cII and cIII function. This inhibition of int synthesis by nucleotide sequences downstream from the int structural gene behaves in a cis-dominant fashion in mixed infections. It is specific for P_1-initiated transcripts. Based on these observations, and those of others, a model is proposed in which int translation from the P_1 transcript is inhibited by the interaction of downstream b2 nucleotide sequences and nucleotide sequences in the int region. The data imply a novel temporal mechanism regulating prophage λ induction; circularization of the prophage genome results in the transposition of inhibitory b2 region sequences next to int and blocks further int protein synthesis beyond the low level required for excision. As a consequence of this process, the control of int expression is transferred from the P_L promoter to P_I and the cII/cIII system. Such a genetic regulatory mechanism involving the rearrangement of genetic elements downstream from a structural gene may be of general use during development in other systems (in collaboration with M. Pearson, FCRC).

The phage and bacterial attachment sites used for λ site-specific recombination (att sites) contain a 15 bp common core region. Recombination occurs within this core region via a 7 bp staggered cut. When a normal phage att recombines with a DNA sequence containing a bacterial att with base changes in the 7 bp overlap region, new att's are generated. We have characterized one novel att site, that when carried by λ , changes the site of integration. The new att site is altered in 3 of the 15 bases in the common core (in collaboration with R. Weisberg, NICHD).

II. We have used recombinant DNA technology and gene fusion techniques to isolate a positive regulatory gene of E. coli. By fusing the lactose operon to a gene controlled by the positive control element, point mutations in the positive regulator can be obtained by simply scoring for lac⁻ phenotypes. We have screened an E. coli. EcoR1 library for hybrid phages that restore the lac+ phenotype. Such phages carry the wild type allele of the positive regulator (in collaboration with T. Silhavy, FCRC).

III. The *EcoR1-H* fragment (15.4 kb) of *herpes simplex* virus type 1 (HSV 1) has been cloned in λ gt*WES* in both orientations. This fragment contains the entire U_S region and has about 900 bp of terminal redundant sequences derived from the internal and terminal repeats of the S region. Fifty-six independent plaque-forming deletion derivatives of the λ gt*WES*. *EcoR1-H* hybrid phage were isolated using either EDTA resistance or ability to grow on *E. coli* P2 lysogens as selective methods. The endpoints of these deletions were located using nine restriction enzymes that cleave in the *EcoR1-H* fragment. All of the deletions have at least one endpoint within the cloned fragment. We have documented several unusual features of the λ hybrids, including heterogeneity of a particular region in the HSV 1 *EcoR1-H* fragment and the presence of Chi-like sequence in the U_S region of HSV-1. Chi sequences stimulate homologous recombination in *E. coli*. The restriction enzyme *Smal* cleaves the *EcoR1-H* fragment at more than 30 sites. We have constructed an *Smal* map of this fragment.

IV. We have begun a collaboration on eukaryotic gene organization using the mouse cytochrome p450's role in chemical carcinogenesis. Using partially purified mouse liver 23S mRNA known to be associated with the *Ah* locus and 3-methylcholanthrene-induced cytochrome P_1-450, we synthesized double-stranded complementary DNA (cDNA) by the successive action of reverse transcriptase (RNA-directed DNA nucleotidyltransferase) and the Klenow A fragment of *E. coli* DNA polymerase I. The double-stranded cDNA was inserted into pBR322 plasmid DNA by *PstI* cleavage and homopolymeric "tailing" and cloned in *E. coli* LE392. Clone 46 hybridizes with [^{32}P]cDNA made from 23S mRNA from "*Ah*-nonresponsive" DBA/2N [^{32}P]cDNA probes; these two clones were therefore used further as "positive" and "negative" control clones, respectively.

By translation arrest experiments, clone 46 DNA and clone 30 DNA are shown to be associated with anti-$P_1$450 and anti-albumin-precipitable material, respectively. By agarose gel electrophoresis of *PstI* digests, the clone 46 DNA insert is shown to be 1,100 bp in total length and to contain one internal *PstI* site. The cDNA made from total mRNA isolated from 3-methylcholanthrene-treated C57BL/6N mice hybridizes to the two fragments of *PstI*-digested DNA from clone 46, whereas similarly prepared cDNA from 3-methylcholanthrene-treated DBA-2N and control C57BL/6N and DBA/2N mice does not. Of eleven restriction endonucleases used, only two (*PstI* and *XbaI*) have sites within the clone 46 DNA insert. Following hybridization of clone 46 ^{32}P-labeled nick-translated DNA to *EcoRI* fragments from A/HeJ mouse genomic DNA, fractionated by RPC-5 chromatography and gel electrophoresis, only one positive band (3 to 4 kbp) appears. These data demonstrate conclusively that pBR322-clone 46 DNA is associated with mRNA controlled by the murine *Ah* locus, presumably the structural gene encoding 3-methylcholanthrene-induced P_1-450 (in collaboration with D. Nebert, NICHD).

Significance to Biomedical Research and the Program of the Institute:

The rearrangement of genetic information by integration of one segment of DNA into another is widespread in prokaryotes and eukaryotes. A specific example is the insertion and excision of viral DNA. Such events are known to change the physiology of the host organisms. It is highly likely that the successful symbiotic relationship of host-virus in the microbial system we employ has an analogy in the relationship between certain animal viruses and the cells of higher organisms. Moreover, an understanding of the control mechanisms operative in these easily studied microorganisms has obvious relevance to an understanding of regulatory elements in higher organisms. The application of recombinant DNA technology and microbial systems to the analysis of the large DNA genome of herpes simplex virus should provide new insight to the biology of this medically important virus. The studies of the cytochrome p450 gene structure will be valuable for understanding the inducible eukaryotic genes involved in chemical carcinogenesis.

Proposed Course:

This project will terminate when the principal investigator leaves government service July 31, 1981.

Publications:

Enquist, L.W.: Application of DNA technology to actinomycetes. *Biol. Actinomycetes and Related Organisms*: 15 (USSR-USA Exchange Conference , Yalta, Crimea, USSR) pp. 7-17, 1980.

Pinkham, J. L., Platt, T., Enquist, L.W., and Weisberg, R.A.: The secondary attachment site for bacteriophage λ in *pro*A/B gene of *E.coli*. *J. Mol. Biol.* 144. 587

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05100-02 LMV

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of Early SV40 RNA and Proteins Synthesized from Ad-2-SV40 Hybrid Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gilbert Jay	Visiting Scientist	LMV, NCI
OTHER:	George Khoury	Acting Chief	LMV, NCI
	Ravi Dhar	Visiting Scientist	LMV, NCI

COOPERATING UNITS (if any)

Dr. Carol Prives, Columbia University, New York.

LAB/BRANCH

Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.4	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In previous studies, we examined hybrid viral RNAs and proteins synthesized in both human and monkey cells infected by non-defective adenovirus SV40 hybrid viruses. A detailed analysis of the hybrid viral transcripts synthesized from AD2+ND4 stocks was performed. These stocks were shown to be nonhomogeneous, consisting of two principle populations of viral DNAs. Both populations contained segments of SV40 DNA extending from map position 0.63 to 0.11 inserted at adenovirus map position 0.79 in a left to right orientation. Thus, they can be transcribed either from the early or late adenovirus promoters. One population, however, contains an intact SV40 DNA segment (about 15% of the molecules), while the major population (85%) contains an SV40 deletion extending from .60 to .50 SV40 map units. This deletion encompasses the SV40 DNA segment encoding early SV40 splice sites; we presume that growth of the viral stock in human cells is inhibited by the presence of these splice junctions. Three major hybrid viral transcripts synthesized late in a lytic infection by these viruses were analyzed in detail. The least abundant species is transcribed from the

minor intact population and contains the SV40 splice junction. A second species extends through the deletion of the major population of the hybrid viruses. The most abundant late population contains a new splice junction extending from a donor site in adenovirus sequences to the novel SV40 acceptor site at 0.46 map units. This SV40 acceptor site is remarkable in that its use has not been detected in spliced SV40 viral mRNA's, either in lytically infected or transformed cells. In addition, we have shown that this novel chimeric splice junction is used only during the late phase (after ND4 DNA replication) of the lytic cycle of this hybrid virus after ND4 DNA replication. When cells were pre-infected by adenovirus 2 for 24 hours and subsequently infected by AD2+ND4 in the presence of cytosine-arabinoside or hydroxyurea (inhibitors of DNA replication), the chimeric splice junction was not detected among the RNA species synthesized. This suggests that the novel splice junction does not depend on a late adenovirus gene product but rather on transcription extending from the AD2+ND4 major late promoter at 0.16 map units. One interpretation of these results is that RNA conformation is important in determining the use of splice junctions.

An analysis of the SV40 T-antigen reactive polypeptides synthesized by the adenovirus SV40 hybrid viruses has been performed. Tryptic peptide analysis has demonstrated the regions of polypeptides represented in proteins synthesized after lytic infection by various hybrid viruses. In addition, both SV40 specific DNA binding properties and nonspecific DNA binding properties have been determined for the polypeptides synthesized by the hybrid viruses. These data, together with tryptic peptide analyses, have suggested that specific segments of the early SV40 gene region encode polypeptides which are directly involved in the binding of the protein to the SV40 genome.

PROJECT DESCRIPTION

Objectives:

To determine the factors involved in RNA splicing and to determine the regions of DNA binding proteins responsible for their specific or nonspecific binding activity.

Methods Employed:

Reverse transcription DNA sequencing, S1 nuclease analysis, Northern transfer technique, tryptic peptide mapping, polypeptide fractionation, DNA binding assays.

Major Findings:

Within the population of hybrid transcripts synthesized in AD2+ND4 infected cells is one which contains a novel viral splice junction. This splice extends from an adenoviral donor sequence to a novel SV40 acceptor site. This splice junction represents (a) the first documentation of a chimeric splice junction, (b) a splice junction which uses a novel SV40 acceptor site not found in pure

SV40 transcripts, and (c) a splice junction used only during the late program of AD2+ND4 infection. It appears likely that the use of this chimeric splice junction at late times depends on RNA conformation rather than a late adenoviral gene product.

The AD2+ND4 hybrid viral polypeptides which react with SV40 anti T serum have been analyzed in detail. These data, together with studies of the DNA binding properties of these polypeptides, suggest that specific regions within the SV40 early genome encode DNA binding segments.

Significance to Biomedical Research and the Program of the Institute:

These studies are significant in terms of models for gene regulation as well as an analysis of the activity of the early SV40 transforming protein T-antigen.

Proposed Course:

These studies have, for the most part, been completed. We will attempt to determine if the novel SV40 acceptor splice junction depends directly on deletion of the principle SV40 acceptor sites. Further studies will be directed at an analysis of the T-antigen DNA binding regions.

Publications:

Khoury, G., Alwine, J., Goldman, N., Gruss, P. and Jay, G.: A new chimeric splice junction in adenovirus-2 SV40 hybrid viral mRNA. J. Virol. 36: 143-151, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05101-03 LMV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Molecular Mechanisms for Malignant Transformation of Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gilbert Jay	Visiting Scientist	LMV, NCI
OTHER:	George Khoury	Acting Chief	LMV, NCI

COOPERATING UNITS (if any)
L. J. Old, Vice President and Associate Director, Memorial Sloan-Kettering Cancer Center, New York, New York.
I. Pastan, Laboratory of Molecular Biology, DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to investigate the molecular mechanisms underlying the malignant transformation of cells. We have been studying the structure and function of tumor antigens involved in neoplastic transformation.

PHS-6040
(Rev. 2-81)

PROJECT DESCRIPTION

Objectives:

Studies on the function of the transformation-specific protein p53 in cancer cells.

Methods Employed:

Proteins were detected either by indirect immunofluorescence or by immunoprecipitation using conventional or monoclonal antibodies.

Major Findings:

We have identified a transformation-related antigen, designated p53, which has the following characteristics:

(a) p53 appears to be a constant feature of tumor cells of the mouse, whether these were transformed by X-ray, chemicals, DNA or RNA viruses or arose spontaneously. In contrast, levels of p53 in normal mouse cells are either extremely low or undetectable.

(b) Immunofluorescence studies have localized p53 to the nucleus of transformed mouse cells.

(c) p53-related components are found in transformed cells of a variety of species, including rat, hamster, rabbit and human. These components vary somewhat in molecular weight, ranging from 53K in mouse cells to 56K in human cells.

(d) Rapidly growing cultures of certain "normal" human cells, such as early passages of kidney epithelium, were found to express p53. However, in contrast to the persistance of p53 in malignant cells when they reach confluence, p53 expression in the normal cell disappears upon contact inhibition of cell proliferation.

(e) In SV40-transformed cells, p53 forms a complex with the viral tumor (T) antigen.

(f) p53 components are immunogenic in their species of origin. Tumors differ, however, in their capacity to induce p53 antibodies.

(g) p53 is a phosphoprotein and has an associated protein kinase activity.

(h) p53 can be induced in normal spleen cells by treatment with lectins.

Significance to Biomedical Research and the Program of the Institute:

The biochemical events responsible for the transformation of normal cells to malignancy have not been defined. A central question in this regard is whether different classes of etiological agents transform cells by uniquely distinct pathways. Certain chemicals, radiations and oncogenic viruses are known to induce neoplasms in laboratory animals. While each of these agents may act distinctly to initiate the transformation of cells, it is possible that once a cell is committed, by whatever mechanism, it will follow a common pathway leading to the loss of control of proliferation. Our identification of a transformation-specific protein common to malignancies of different etiologies allows us to test this hypothesis and provides us with a biochemical handle to study the molecular basis for transformation.

Proposed Course:

Attempts will be made to define the molecular mode of action of this protein during the process of malignant transformation. The possible application of our findings to clinical diagnosis and therapy will be entertained.

Publications:

DeLeo, A. B., Jay, G., Appella, E., Dubois, G. C., Law, L. W., and Old, L. J.: In: Alien Histocompatibility Antigens and Cancer Cells. Ed. M. M. Bortin and R. L. Turner. Grune and Stratton, New York, 1980.

DeLeo, A. B., Jay, G., Appella, E., Dubois, G. C., Law, L. W., and Old, L. J.: Cell surface antigens of chemically induced sarcomas of inbred mice. Transplant. Proc. 7: 65-69, 1980.

Jay, G. and Khoury, G.: Induction of a unique antigen upon malignant transformation. In: Leukemias, Lymphomas and Papillomas: Comparative Aspects. Ed. P. A. Bachman. Taylor and Francis, 1980.

Yasamoto, T., Jay, G., and Pastan, I.: Unusual features in the nucleotide sequence of a cDNA clone derived from the common region of the avian sarcoma virus messenger RNA. Proc. Natl. Acad. Sci. USA 77: 176-180, 1980.

Yasamoto, T., Sivaswami-Tyagi, J., Fagan, J. B.,, Jay, G., deCrombrugghe, B., and Pastan, I.: Molecular mechanism for the capture and excision of the transforming gene of avian sarcoma virus as suggested by analysis of recombinant clones. J. Virol. 35: 436-443, 1980.

Dippold, W. G., Jay, G., DeLeo, A. B., Khoury, G., and Old, L. J.: p53 transformation-related protein: detection by monoclonal antibody in mouse and human cells. Proc. Natl. Acad. Sci. USA 78: 1695-1699, 1981.

Jay, G., Khoury, G., DeLeo, A. B., Dippold, W. G., and Old, L. J.: p53 transformation-related protein: detection of an associated phosphotransferase activity. Proc. Natl. Acad. Sci. USA 78: 2932-2936, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05102-03 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Generation of HSV-1 Defective DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Katherine Denniston	Senior Staff Fellow	LMV, NCI
OTHER:	L. W. Enquist	Research Scientist (PHS)	LMV, NCI
	G. F. Vande Woude	Chief, VTBS	LMV, NCI

COOPERATING UNITS (if any)
George H. Brownell, Associate Professor, Dept. of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30902

LAB/BRANCH
Laboratory of Molecular Biology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Detailed physical maps of five atypical cloned DNA fragments from defective interfering particles of herpes simplex virus type 1 (HSV-1) strain Patton were constructed. Unlike typical cloned fragments of defective HSV-1, these fragments have non-contiguous HSV-1 DNA fused to S region terminal repeat sequences (TR_S). While four of the five fragments contained only S region sequences (grossly rearranged), one contained distant U_L sequences fused to a portion of TR_S.

PHS-6040
(Rev. 2-81)

PROJECT DESCRIPTION

Objectives:

To determine the origin of HSV-1 dDNA fragments in the wild-type HSV-1 genome and the structure of specific dDNA cloned fragments. These analyses can reveal how defective molecules of HSV are generated. The cloned dDNA fragments are used also to investigate the replication of herpes simplex virus.

Methods Employed:

Four methods are being used to study the fine structure of the HSV dDNA clones. The first is restriction endonuclease mapping of dDNA clones and of cloned DNA from the same region of the wild-type HSV genome. These large dDNA EcoRl fragments are then subcloned, using other restriction enzyme sites, from the lambda phage vector into the plasmid vector pBR322. These small subclones enable determination of maps for restriction emzymes having 10-30 cut sites. Such detailed restriction maps allow the determination of the nucleotide sequence of the interesting regions of the dDNA molecule. Both the Maxam and Gilbert chemical degradations, as well as the newer nick translation and chain termination enzymatic sequencing techniques, will be used. Southern transfer hybridization experiments are used to study the origin of HSV-1 dDNA.

Major Findings:

Fine structure mapping techniques using acrylamide-agarose gels and DBM-blotting were developed to understand several atypical cloned HSV-1 defective DNA fragments. This technology enabled us to show that the atypical fragments were of the same general structure as normal HSV-1 defective DNA, but were riddled with deletions. One clone contained an unusual fusion of the TR_S sequences and 2000 bp of DNA some 80,000 bp distant.

The DNA of Actinophage ØEC was characterized by physical and enzymatic methods. In the electron microscope, the DNA appeared as a linear, double-strand molecule with an average chain length of 43.2 + 7 kbp. Further studies suggested that the DNA carried cohesive ends. The DNA was analyzed by digestion with 32 restriction endonucleases. The enzymes BamHI, HpaI, PvuII, XbaI and XhoI were used to construct a map of the ØEC genome.

Significance to Biomedical Research and the Program of the Institute:

The portion of the genome which gives rise to dDNA has been shown to activate the expression of endogenous type C virus genome in mouse cells. This region also accelerates malignant transformation of mouse cells. The region contains putative replication and encapsidation signals as well as several strong immediate early promoters. An understanding of the DNA arrangements within this region will lend insight into the function and control of HSV-1 replication.

Proposed Course:

This project was terminated in March 1981 when Dr. Denniston left the NIH.

Bibliography:

Brownell, G. H., Enquist, L. W. and Denniston-Thompson, K.: An analysis of the genome of actinophage ØEC. Gene 12: 320-325, 1980.

Denniston, K. J., Madden, M. J., Enquist, L. W. and Vande Woude, G.: Characterization of coliphage lambda hybrids carrying DNA fragments from herpes simplex virus type I defective interfering particles. Gene, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05103-03 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure and Function of MSV Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William McClements	Senior Staff Fellow	LMV, NCI
Other:	G. F. Vande Woude	Chief, VTBS	LMV, NCI
	D. G. Blair	Expert	LVC, NCI
	M. L. McGeady	NIH Fellow	LMV, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure and biology of cloned Moloney sarcoma provirus has been characterized. From cloned proviral subgenomic fragments, we have identified sequences required for efficient transformation and for generation of rescueable transforming virus. The retrovirus long terminal repeat (LTR) is required in addition to the acquired cellular sequence (mos) for efficient transformation. The LTR is also capable of activating the cellular homolog of viral mos. Cells transformed by subgenomic fragments containing an intact 5' end of the MSV LTR through c-mos contain a rescueable transforming virus. These findings led to the development of potential retrovirus vectors for activation and rescue of cellular sequence with transforming potential. Prototype retrovirus cloning vehicles based on MSV sequences have been sucessfully tested in model systems.

PHS-6040
(Rev. 2-81)

PROJECT DESCRIPTION

Objectives:

This project aims to characterize the components of the proviral genome of Moloney sarcoma virus (MSV) that are a) responsible for efficient cellular transformation and b) essential for virus rescue. Sequences isolated from cloned proviral MSV DNA are used to activate and rescue transforming sequences from the normal eukaryote chromosome.

Methods Employed:

DNA from normal or transformed cells is digested with restriction endonucleases and analyzed by the Southern blotting technique. Recombinant DNA technology is employed to clone retrovirus proviral DNA subgenomic fragments and normal cellular DNA fragments. Biological activity of cloned fragments is determined in a direct DNA transfection assay. RNA from transformed cells and virus rescued from those cells is analyzed by Northern blotting techniques.

Major Findings:

The integrated proviruses of two Moloney sarcoma virus (MSV) isolates have been molecularly cloned and studied in detail. Clones containing subgenomic fragments of the proviruses have been examined for biological activity in an effort to determine which proviral sequences are required for transformation. The complete provirus transforms NIH 3T3 cells with efficiencies of 40,000-50,000 focus-forming units per picomole of transfected DNA. MSV can be rescued from most of these transformed cells. A cloned internal fragment containing 1,200 base-pairs (bp) of the sarcoma-specific acquired cellular sequence (v-mos) and $\sim$500 bp of leukemia virus derived sequences 5' to v-mos transforms cells about 10,000 times less efficiently than the intact provirus. If one long terminal repeat (LTR) of the provirus is linked either 5' or 3' to v-mos, transformation is stimulated $\sim$1,000 fold. The LTR can also stimulate transformation by v-mos when an LTR-bearing clone is co-transfected with v-mos. The cloned cellular homolog of v-mos, called c-mos does not transform cells in this assay. But when linked to an LTR, the transforming potential of c-mos is activated (Z01 CP 04882-06 LMV). Thus, the LTR is essential for efficient transformation by mos. The LTR contains putative RNA transcription control signals that may allow expression of mos mRNA. Analysis of polyadenylated RNA from cells transformed with subgenomic clones of proviruses is consistent with evidence that LTR provides transcription control signals (Z01-CP-05156-01-LMV). The LTR has been shown to have structural features and biological activities in common with prokaryotic and eukaryotic transposable elements. These features, and the ability to activate the transforming potential of a normal cellular gene, raise the possibility that LTR-like sequences may play a role in a variety of neoplastic transformations.

Cells transformed by subgenomic clones of MSV containing an intact 5' end of the provirus, including the left LTR, gag sequences and v-mos, contain rescueable transforming retroviruses. This observation has led to the use of sequences from the 5'end of the provirus (excluding v-mos) as vectors to identify and clone other cellular sequences with transforming potential. These retroviral vectors supply an LTR to activate linked gene expression and provide viral sequences necessary to form a rescueable retrovirus. Model systems to test the efficacy of such vectors have shown that it is possible to stimulate transformation by a transforming sequence and to rescue a transforming virus.

Significance

These studies have shown that transforming activity of the acute transforming retroviruses is due to the combination of a sequence with transforming potential, and the retrovirus LTR, which allows high levels of expression of the transforming sequence. Direct activation of the oncogenic potential of a normal cell sequence (c-*mos*) by an LTR suggests a model for at least some forms of oncogenesis. LTRs or LTR-like elements, when juxtaposed with cellular genes with transforming potential may activate them. The development of vectors that can activate and rescue cellular sequences will allow a method of identifying a variety of genes with oncogenic potential.

Proposed Course:

The retroviral cloning vectors will be further refined and tested extensively with both cloned and uncloned transforming genes. Eukaryotic genomic DNA, including that from humans, will be used as the source of potential transforming genes to be activated and rescued by the vectors.

Publications:

Blair, D.G., McClements, W.L., Oskarsson, M. and Vande Woude, G.F. Properties of the Moloney leukemia virus genome required for efficient transformation in *mos*. In *Modern Trends in Leukemia Research*, Springer-Verlag. In Press.

McClements, W. Retrovirus Integration, in *Microbiology 1981*, in press.

McClements, W.L., Dhar, R., Blair, D.G., Enquist, L., Oskarsson, M. and Vande Woude, G.F. The long terminal repeats of integrated Moloney sarcoma provirus are like bacterial insertion sequences (IS) elements. *Cold Spring Harbor Symp. on Quant. Biol.*, Vol. *45*, 1981. In Press.

McClements, W.L. and Vande Woude, G.F. Cloning Retroviruses: Retrovirus Cloning? *Genetic Engineering, Vol. III*, Plenum Press, pp. 699-705, 1981.

Blair, D.G., Oskarsson, M.K., Wood, T.G., McClements, W.L., Fischinger, P.J. and Vande Woude, G.F. Activation of the transforming potential of a normal cell sequence: A molecular model for oncogenesis. *Science 212*: 941-943, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05104-02 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Splicing in Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter Gruss	Expert	LMV, NCI
OTHER:	George Khoury	Acting Chief	LMV, NCI

COOPERATING UNITS (if any)

Dr. Argiris Efstratiadis, Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an attempt to determine the function of the intron, we have generated a deletion mutant lacking precisely the intron of SV40 16S mRNA. This SV40 deletion mutant produced no stable late viral RNA, which suggested a defect is post-transcriptional processing of the viral RNA. Subsequently, we used this mutant as the recipient for the insertion of an isolated mouse β^{maj} globin intron. Cloned recombinants harboring the inserted intron in the sense direction give rise to stable mRNA. These observations suggested that introns represent functional elements in the generation of certain mRNAs.

On the other hand, the recent detection of a number of eukaryotic genes which are uninterrupted by intervening sequences, as well as certain late mutants of SV40, which appear to produce unspliced 19S late mRNAs, have suggested that alternative mechanisms must exist for the expression of nonspliced transcripts.

PHS-6040
(Rev. 2-81)

In an attempt to shed further light on this question, we constructed a recombinant DNA molecule carrying the coding region and the 3' flanking sequences of the rat preproinsulin I gene, replacing the late region of an SV40 viral vector. The sequences comprising the single intron of the preproinsulin I gene, located within the nontranslated leader region, as well as all of the 5'-flanking sequences, have been deleted. The sequences remaining within the late transcriptional unit of SV40 do not retain a functional intron. This "intronless" mutant nevertheless directs the synthesis of substantial quantities of unspliced stable hybrid transcripts which are translated into polypeptides with insulin immunoreactivity. Two novel 5' termini, not used in wild-type SV40 transcripts, have been detected in the majority of the hybrid RNA molecules. These are located at analogous positions in both of the late SV40 72 base-pair repeats.

PROJECT DESCRIPTION

Objectives:

The project aims to study gene expression in mammalian cells using SV40 as a vector for the insertion of cloned eukaryotic genes, or portions thereof, in order to elucidate transcriptional or post-transcriptional mechanisms for the expression of the inserted genes. The main objective is construction of recombinant molecules with subsequent deletion of essential regions to determine nucleotide sequences involved in initiation and splicing of the transcripts.

Methods Employed:

Construction of recombinant molecules: restriction endonuclease excision of DNA fragments, modification of the ends by addition of oligonucleotide "linkers;" ligation of required DNA fragments, cloning of altered genomes by complementation with helper virus. Mapping of the recombinant genomes: analysis with restriction endonucleases. RNA-analysis: S-1 nuclease technique and a modification of the Exo7 nuclease method, "Northern" blots, reverse transcription and sequence analysis, immunoprecipitations of proteins, electrophoresis in SDS-urea polyacrylamide gels, cloning of DNA fragments in pBR322, and single-strand bacteriophage fd.

Major Findings:

The role of RNA splicing in the post-transcriptional processing of mRNA precursors is under intense investigation. Previous experiments by several groups have suggested that certain transcripts, generated either from SV40 recombinant molecules or from mutants of the viral genome, require splicing for their stable expression in the cytoplasm. On the other hand, the recent description of a number of eukaryotic genes which are uninterrupted by intervening sequences have suggested that alternative mechanisms must exist for the expression of unspliced transcripts. We addressed this question by employing SV40 rat preproinsulin recombinant molecules which retain a single rat preproinsulin intervening sequence in the late region of the genome. While all of the late recombinant transcripts appeared to use this intron in the preproinsulin sequence as a splice site, it was unclear whether this particular splicing event was obligatory to the production of stable mRNA. Mutants of this recombinant molecule were constructed in which the unique preproinsulin DNA intron, as well as the flanking sequences 5' to the gene, were removed. In the new recombinant molecule, the first five codons of the preproinsulin coding sequence were deleted and replaced "in frame" with the first three codons of the SV40 agnoprotein. This resulted in the fusion of the coding region for the rat chromosomal preproinsulin I gene to the 5' noncoding region of the late SV40 genes. While this construction deleted potential 5' ends of the rat preproinsulin transcripts, the region of SV40 which contains sequences encoding the 5' late mRNA ends was preserved and was situated in the immediate vicinity of the new junction between the sequences of the vector and the insert.

Both RNA and protein analyses revealed that the new mutant recombinant molecules were capable of generating stable hybrid co-linear transcripts lacking splice junctions. This finding was unexpected, since earlier constructions of SV40 deletion mutants and recombinant molecules with foreign DNA fragments appeared to require a splicing event for production of a stable mRNA. An associated, and perhaps related, finding was the absence of RNA molecules containing the 5' termini generally recognized as the most abundant class of wild-type late SV40 transcripts. Instead, the two most common 5' ends were situated in analagous sites in the late repeated sequences of SV40 exactly 72 base-pairs apart. These are, in fact, identical to certain 5' ends detected in unspliced RNA molecules generated by a viable SV40 late deletion mutant. In view of these results, one might suggest a correlation between the location of the SV40 RNA 5' end and the presence or absence of splicing in the stable transcripts. How the position of the RNA termini might relate to the requirement for splicing is not at all clear. One possibility is that these altered unspliced transcripts contain new sequences or sequence modifications which permit the novel RNA molecules to bypass a putative splicing requirement and yet retain their stability. An alternative possibility is that the size or secondary structure of a particular transcript or its destination for free or membrane bound polysomes in part determines the potential requirement for splicing in its biogenesis. While we have shown in this study that stable non-spliced hybrid RNA molecules can be synthesized from the late region of SV40 recombinants, the relationship of splicing and stability of certain mRNAs such as the late SV40 16S species still remains obscure.

Significance to Biomedical Research and the Program of the Institute:

Eukaryotes have been found to be comprised mostly of genes which are noncolinear with their mRNA's. The role of the intervening sequence for the expression of eukaryotic genes is of importance for an understanding of the stable expression of eukaryotic genes.

Proposed Course:

This project will continue with attempts to decipher the genetic signals required for the expression of unspliced genes.

Publications:

Gruss, P. and Khoury, G.: Rescue of a splicing defective mutant by insertion of a heterologous intron. Nature 286: 634-637, 1980.

Gruss, P., Lai, C.-J., Dhar, R. and Khoury, G.: Splicing as a requirement for biogenesis of functional SV40 mRNA. Proc. Natl. Acad. Sci. USA 76: 4317-4321, 1980.

Gruss, P., Efstradiatis, A., Karathanasis, S., Konig, M., and Khoury, G.: Synthesis of unspliced mRNA from an SV40-rat preproinsulin recombinant lacking the intron. Proc. Natl. Acad. Sci. USA, 1981, in press.

Gruss, P., and Khoury, G.: Gene transfer into mammalian cells: Use of viral vectors to investigate regulatory signals for the expression of eukaryotic genes. In Goebel, W., and Hofschneider, P.H., Current Topics of Microbiology and Immunology. Springer-Verlag, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05151-02 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
SV40 DNA Replication In Vitro and Cellular Factors Involved in Host Range

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Chikako Sumida-Yasumoto	Expert	LMV, NCI
OTHER:	George Khoury	Acting Chief	LMV, NCI

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Research Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To understand the biochemical mechanisms of eukaryotic DNA replication, we have developed a unique soluble cell-free system which catalyzes SV40 DNA replication dependent on exogenously added SV40 form I DNA and SV40 large T-antigen. The system is characterized by de novo initiation of SV40 DNA replication in vitro. An in vitro complementation assay for SV40 large T-antigen based on this in vitro SV40 DNA replication showed that the extract prepared from SV40-infected CV-1 cells did contain a functional SV40 large T-antigen, whereas the extract prepared from SV80 cells (a SV40 transformed human cell line) did not contain a functional large T-antigen. Furthermore, the extracts prepared from the rapidly growing (log phase) CV-1 cells which were not infected with SV40, also supported SV40 DNA replication in vitro even in the absence of SV40 large T-antigen. Extracts prepared from uninfected stationary (G_0 phase) CV-1 cultures did not support SV40 DNA replication.

In vivo, a T antigen deletion mutant of SV40 appears to replicate its DNA in the rapidly growing log phase CV-1 cells, but not in the stationary culture. One explanation for these results is the presence of a cellular protein(s), whose function might be equivalent to SV40 large T-antigen. Such putative proteins may exist only in the rapidly growing (log phase) uninfected CV-1 cells, but not in the uninfected stationary cells.

PROJECT DESCRIPTION

Objectives:

To understand the biochemical mechanisms of eukaryotic DNA replication by studying in vitro SV40 DNA replication as a model system. To assay for cellular proteins which may bear some functional similarity to SV40 T-antigen.

Methods Employed:

Sucrose gradient centrifugation; isopycnic centrifugation; in vitro DNA replication assay; in vitro complementation assay for SV40 large T-antigen; DNA-DNA hybridization; agarose and acrylamide gel electrophoresis; restriction endonuclease digestion analysis of DNA.

Major Findings:

1. Analysis of in vitro SV40 DNA replication was established.

   (a) Preliminary kinetic analysis of in vitro SV40 DNA products by DNA-DNA hybridization suggested that in vitro DNA replication occurred bidirectionally from the SV40 DNA replication origin.

   (b) The in vitro complementation assay for SV40 large T-antigen based on in vitro SV40 DNA replication showed that the extract prepared from SV40-infected CV-1 cells did contain functional large T-antigen, whereas the extract prepared from SV80 cells (an SV40 transformed human cell line which over-produces SV40 large T-antigen which has been employed as one of the main sources for SV40 large T-antigen) did not contain a functional SV40 large T-antigen.

   (c) An extract prepared from the rapidly growing (log phase) uninfected CV-1 cells also supported SV40 DNA replication in vitro even in the absence of SV40 large T-antigen. A similar extract prepared from the uninfected stationary (G_0 phase) CV-1 cultures did not support SV40 DNA replication. Similarly, in vivo, a T-antigen deletion mutant of SV40 replicated its DNA in the rapidly growing log phase CV-1 cells, but not in a stationary culture. Thus, a cellular protein(s), whose function is equivalent to SV40 large T-antigen, might exist in the rapidly growing uninfected CV-1 cells but not in the uninfected stationary cells.

2. The effects of four E. coli gyrase inhibitors, novobiocin,,coumermycin A_1, nalidixic acid, and oxolinic acid on eukaryotic DNA replication were studied. These agents were shown to inhibit DNA replication of both the host monkey kidney cells, as well as that of SV40 which had been inoculated into these cells. Furthermore, SV40 DNA synthesis in the soluble in vitro system (which depends on the SV40 gene A product large T-antigen and exogenously added SV40 form I DNA as a template) is sensitive to these inhibitors. Results of experiments in the monkey kidney cells indicate that SV40 DNA replication is inhibited at least at the step of elongation by the four drugs; this inhibition results in a partial relaxation of SV40 form I DNA.

Significance to Biomedical Research and the Program of the Institute:

The study of the mechanism of mammalian chromosome replication will provide us one of the answers for the mechanism of cell transformation as well as host range of DNA tumor viruses.

Proposed Course:

More detailed study of in vitro SV40 DNA replication to understand the biochemical mechanisms of eukaryotic DNA replication is proposed.

Purification and characterization of the multi-functional protein, SV40 large T-antigen, and a cellular protein(s) which may have a similar function will be pursued.

Publications:

NONE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05152-02 LMV

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of the Functional Interaction of Oncogenic DNA Virus Gene Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Neil Goldman	Staff Fellow	LMV, NCI
OTHER:	George Khoury	Acting Chief	LMV, NCI
	Barbara Dunn	Guest Worker	LMV, NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Experiments were carried out to analyze functional interactions between early viral proteins of oncogenic DNA viruses from different groups. The SV40 A gene product (T antigen) could complement a mutant of human adenovirus temperature sensitive DNA replication in semi-permissive monkey cells at the non-permissive temperature. Early proteins from adenovirus (presumably the 72 Kd early Ad 2 protein) could regulate the over production of SV40 early mRNA synthesis by tsA mutant of SV40. Low stringency hybridization experiments indicated that limited DNA sequence homology occurred between SV40 and adenovirus at control regions for replication and transcription, where the SV40 T antigen and adenovirus 72 Kd protein bind.

We have also found a novel modification, poly ADP-ribosylation, present in a minor population of T-antigen. The absence or presence of this modification may be important for replication. These findings suggest that there can be mutual interaction of viral proteins between viruses of diffrent groups which function at the level of replication and transcription. Future work includes

molecular cloning into a papilloma virus DNA vector of the early region of SV40 which includes the origin, "promoters" and coding region of T antigens. This has been undertaken to identify further the role of thse DNA sequences and proteins in integration, transformation and gene expression.

PROJECT DESCRIPTION

Objectives:

To study the structural and functional interactions of tumor virus gene products between viruses of different groups.

Methods Employed:

DNA, RNA and protein extractions, polyacrylamide and agarose gel electrophoresis of DNA and proteins, restriction enzyme cleavage of DNA and transfer by Southern blotting technique, hybridization of DNA or RNA to DNA containing blots, immunoprecipitation of proteins, thin-layer chromatography of nucleotides.

Major Findings:

Our previous findings indicated that human adenovirus is able to inhibit SV40 DNA replication during co-infection of monkey cells without affecting SV40 transcription or translation. Similar studies had determined that the inhibition of replication was not simply due to competition for enzymes, enzyme sites or cellular metabolites. We hypothesized that adenovirus competes directly with SV40 for an anabolic precursor essential for replication--a prime candidate is SV40 T antigen (the protein specifically required for SV40 replication). We found that, indeed, an adenovirus conditional lethal replication mutant could be complemented with wild-type SV40, but not with an early SV40 mutant encoding a temperature-sensitive T antigen. This suggested the ability of adenovirus to sequester and utilize T antigen. We also found the converse, that wild-type adenovirus (presumably 72K protein) could modulate the overproduction of early SV40 transcription by a temperature-sensitive SV40 mutant approach wild-type SV40 levels. SV40 early transcription is regulated by T antigen.

Since T antigen, a single polypeptide, plays a role in many diverse functions including SV40 replication, transcription transformation, and the adenovirus helper effect, it was plausible that a particular activity could be regulated by a post-translational modification of the polypeptide. It was previously shown that T antigen can be phosphylated and acetylated. We have found that a minor population of wild-type T antigen is ADP-ribosylated and that temperature-sensitive T antigen is modified at lower levels. This may suggest a role for this modification in a specific T-antigen function.

We are also cloning the origin, promoters and early coding region of SV40 into a papilloma virus vector to study the effects on integration, transformation and gene expression. Preliminary evidence indicates a substantial increase in the efficiency of transformation of the chimeric vector and coincident expression of SV40 large and small T antigen.

Significance to Biomedical Research and the Program of the Institute:

These experiments have been directed toward studying the ability of viral tumor antigens to interact in heterologous systems and to influence macromolecular functions including replication, transcription, transformation and tumor specific transplantation immunity.

Proposed Course:

This study will be continued with an emphasis on the role of the controlling region of DNA around the SV40 origin of replication and the early gene products (T antigens) on transformation, integration and gene expression.

Publications:

Goldman, N., Brown, M., and Khoury, G.: Modification of SV40 T antigen by poly ADP-ribosylation. Cell 24: 567-572, 1981.

Goldman, N., Howley, P. and Khoury G.: Functional interaction between the early viral proteins of simian virus 40 and adenovirus. Virology 109: 303-313, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05156-02 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Expression of MSV Genetic Information

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomas Gordon Wood	Senior Staff Fellow	LMV NCI
OTHER:	G.F. Vande Woude	Chief VTBS	LMV NCI
	W.L. McClements	Senior Staff Fellow	LMV NCI
	M.L. McGeady	Post-Doctoral Fellow	LMV NCI
	D.G. Blair	Expert	LVC NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Polyadenylated RNA from cells transformed by transfection with subgenomic proviral DNAs of ml and HT-1 Moloney murine sarcoma virus (M-MSV) were analyzed for mos containing RNA. Cells transformed by subgenomic proviral DNAs containing a 5' LTR relative to the v-mos sequence were found to exhibit identical RNA patterns when Northern blots of these RNAs were analyzed with probes representing mos and the unique 5' region of the proviral LTR (U5). These RNAs did not contain sequences hybridizable to the probe prepared from the unique 3' region of the LTR (U3). The size of the transcripts containing mos were in excess of the DNA used in the transfection and imply the involvement of host or carrier DNA sequences in these transcripts. Poladenylated RNA, isolated from cells transformed by transfection with a subgenomic proviral DNA in which the LTR was 3' relative to v-mos, showed identical RNA patterns with U3 and mos probes, but not with U5. Analysis of polyadenylated RNA from cells transfected with an LTR: c-mos hybrid molecule showed several mos-specific RNAs and demonstrate that c-mos is activated for expression by the proviral LTR sequence.

Objectives:

To identify and characterize sequences responsible for neoplastic transformation. The specific goals are to identify genetic regulatory sequences present in the retroviral long terminal repeat (LTR) and to assess their specific function in expression of oncogenic messenger RNA. Utilizing recombinant DNA technology, biologically active proviral DNA from two separate strains (ml and H T-1) of Moloney murine sarcoma virus (M-MSV) were cloned. Subgenomic clones of M-MSV were analyzed in transfection assays to determine those segments of the proviral genome responsible for the neoplastic capabilities of these viruses. In addition, the cloned v-*mos* sequence was used to identify and isolate its cellular homolog, c-*mos*, from normal mouse DNA. While subgenomic clones of M-MSV containing v-*mos* were active in transfection-transformation assays, cloned cellular DNA containing c-*mos* was not. Activation of the transforming potential of c-*mos* was accomplished by linking a proviral LTR 5' to the c-*mos* sequence. Many of the subgenomic clones constructed for these experiments contained only a single LTR covalently linked either 5' or 3' relative to the *mos* sequence present in the respective clone. Expression of these DNA's may require additional regulatory sequences, presumably, supplied by either the host or carrier DNA during the transfection. The long range goals of this research are to extend the analysis of transcriptional control signals to candidate cellular promotors and terminators sequestered during the transfection process by the transforming DNA. By cloning these DNA sequences back from the cells transformed in the transfection assays and applying similar reconstruction experiments as used in the analysis of the subgenomic proviral DNAs, an ultimate understanding of the mechanisms involved in regulating eukaryotic gene expression is possible.

Methods Employed:

Polysomal RNA is isolated from normal, viral-infected and DNA-transfected cells and poly(A) containing RNA selected by affinity chromatography using oligo (d T) cellulose. RNA species are separated by electrophoresis on agarose-CH_3 Hg OH gels and analyzed by Northern blotting techniques. Radioactive probes specific for various DNA segments which comprize the transfecting DNAs are prepared by restriction endonuclease digestion of subgenomic proviral DNA and separation of the DNA fragments by preparative polyacrylamide gel electrophoresis. Where necessary, secondary restriction endonuclease digestion and polyacrylamide gel electrophoresis is employed to yield a highly purified DNA for labeling with ^{32}P-dNTP's in a nick translation reaction. To identify those RNA's that are the result of RNA splicing or to ascertain the amount of a given DNA sequence being expressed in the RNA transcripts, RNAs are hybridized to various well-characterized DNAs in a DNA excess reaction. Following completion of the hybridization reaction, the mixture is subjected to either S1 or exonuclease VII digestion and then subjected to electrophoresis on NaOH agarose gels and analyzed by Southern blotting technique using the appropriate probes. DNA from cells transformed in transfection assays using subgenomic DNA clones is isolated, digested with restriction endonucleases, subjected to agarose gel electrophoresis and analyzed by Southern blotting technique to determine candidate DNA segments to be used in isolating cellular regulatory sequences. Recombinant DNA technology is employed to clone selected DNAs for further analysis.

Major Findings:

1. Identified the viral specific RNAs in cells transformed by five separate strains of M-MSV.

2. Identified a candidate subgenomic mos-specific mRNA in HT-1 transformed Crandel cat cells.

3. Identified and partially characterized the mos-specific RNA transcripts present in NIH 3T3 cells transformed by transfection with subgenomic ml and HT-1 proviral DNAs. Clones containing a single LTR at either the 5' or 3' position relative to the v-mos sequence were analyzed. While the number and size of the RNA transcripts varied from foci to foci and with the transfecting DNA, clones containing a 5' LTR relative to v-mos contained RNA transcripts which were equally recognized by probes to mos and the U5 region of the LTR, but not the U3 region. Conversely, cells transfected with subgenomic DNA in which the LTR was 3' to v-mos contained RNA patterns identical when analyzed with mos or U3 probes, but these same RNA transcripts did not contain sequences hybridizable with U5 probe. In many cases, the size of the RNA transcript was in excess of the DNA used in the transfection analysis implicating the involvement of host or carrier DNA sequences in these transcripts.

Significance to Biomedical Research and the Program of the Institute:

There are at least twelve known oncogenes that have been transduced by retroviruses. For many of these sequences, a normal cellular gene has been identified. These cellular homologs are apparently not as effective as their viral counterparts in their ability to transform cells, suggesting that their expression is tightly requlated in the normal cell. Obviously, to ascertain what will alter this quiescent state and result in oncogenic expression would be of paramount importance to our understanding of neoplastic disease. Currently we know that the retroviral LTR contains all the necessary information to activate the murine oncogene c-mos. Future experiments will, hopefully, extend this observation to other oncogenes. A detailed analysis of the various transcriptional control elements contained within the retroviral LTR will aid in evaluating the basic requirements of gene expression and by using mos: using LTR DNA hybrids, we intend to extend our analysis to normal cellular promotor and terminator sequences involved in gene expression.

Publications:

Ng, V.L. Kopchick, J.J. Karshin, W.L., Wood, T.G. and Arlinghaus, R.B. The structural relatedness of the virus core proteins of Rauscher and Moloney murine leukemia virus. *J. Gen. Virol.* 47, 161-170, 1980.

Wood, T.G., Somers, K. and Arlinghaus, R.B. Isolation and partial characterization of a 55,000 - Dalton protein induced in cells transformed by the ml strain of Moloney murine sarcoma virus. *Virol*. 105: 148-158, 1980.

Arlinghaus, R.B., Murphy, E.C., Jr., Lyons, D.D., Horn, J.P., Mong, S-M. and Wood, T.G. Translation products of the 124 strain of Moloney murine sarcoma virus (Mo-MSV): Characterization of a 23,000 Dalton candidate *src* gene product. In Fields, Bernard; Jaenisch, Rudolf; Fox, C. Fred (Eds.): *Animal Virus Genetics. ICN-UCLA Symposia on Molecular and Cellular Biology*, Vol. SVIII. New York, Academic Press, 1980, pp. 643-655.

Blair, D.G., Oskarsson, M.K., Wood, T.G., McClements, W.L., Fischinger, P.J. and Vande Woude, G.F. Activation of the transforming potential of a normal cell sequence: A molecular model for oncogenesis. *Science* 212: 941-943, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CO 05212-01 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Manipulation of Cloned HSV DNA Fragments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lynn W. Enquist	Research Scientist (PHS)	LMV, NCI
Other:	Anamaris M. Colberg-Poley	Postdoctoral Fellow	LMV, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genetics of herpes simplex virus type 1 (HSV-1) is being examined using cloned HSV sequences. The initial portion of these studies has established the feasibility of introducing cloned HSV sequences into infectious virus by cotransfection. We are presently studying novel HSV recombinants which we have generated in alternate vectors (simian virus 40 [SV40] and bacteriophage λ 1059). These cloned sequences will be available for mutagenesis and studies of gene expression in the latter course of our experiments.

PROJECT DESCRIPTION

Objectives:

Our general objective is to study how HSV-1 regulates the expression of its genome. Specifically, we will analyze a specific region (S) by generating mutants in this region following *in vitro* mutagenesis of cloned HSV sequences and reinsertion into infectious virus and by examining the expression of isolated HSV genes in recipient (Xenopus or mammalian) cells.

Methods Employed:

The introduction of HSV-1 markers from cloned sequences into infectious virus was accomplished by cotransfections of a known temperature sensitive (ts) mutant DNA (HSV-1, KOS, ts B21u) and recombinant DNAs containing appropriate HSV-1 (Patton) sequences. The products of the cotransfections were assayed by plaque formation at permissive and nonpermissive temperatures.

To facilitate the expression studies, HSV-1 DNA which codes for an immediate early mRNA was isolated after restriction cleavage and was ligated to a eukaryotic vector (SV40) DNA. The SV40 DNA used in the ligations spans from 0.14 to 0.72 map units and includes all the SV40 early region. The ligation mixture was transfected in presence of helper virus (SV40 tsA) at nonpermissive temperature. The plaque isolates were grown, ^{32}P-DNAS extracted by modified Hirt procedure, and hybridized to Southern blots of HSV-1 DNA. Some of the HSV-containing SV40 hybrids were grown and larger quantities of DNA (from cleavage of a recombinant plasmid, pkL43) were purified by banding on cesium chloride gradients. The purified DNAs were analyzed by cleavage with restriction endonucleases.

To obtain HSV-1 (KOS) DNA in large quantities for future studies, cloning with a λ vector which accepts Bam HI fragments from 6t.3 to 24.4 K6 was performed. Large partial digestion products of HSV-1 (KOS) DNA cut with Sau 3AI were isolated and ligated to Bam HI cleaved λ1059. The ligation products were *in vitro* packaged and plated on an *E. coli* P2 lysogen. The recombinants were isolated and DNA extracted from pelleted virions. The recombinants are being mapped using restriction enzyme cleavages and by hybridization to cleaved HSV-1 KOS DNA.

Major Findings:

The lesion in ts B21u has been previously found to map within the terminal *Eco*RI "K" sequences. These sequences are present within both TRs and Ihs. Our results confirm its position. tsB21u was rescued by recombinants containing portions of "K" from defective (12-7, 12-11, and 1-1) and nondefective (pRWA-1) DNAS. However, plasmids containing TRs sequences but not *Eco*RI "K" (pKL43) or containing only Us (127-2) were not capable of rescuing tsB21u.

Control experiments show that vector DNAs (λ or PBR 325) are not capable of rescuing tsB21u. From these studies, we conclude that recombinant molecules of HSV-1 defective and nondefective genomes contain authentic HSV sequences, and these cloned sequences are capable of recombining with tsB21u generating infectious virus lacking ts lesion.

Restriction analysis of the SV40-HSV hybrids generated in these studies were found to contain rearrangements; the expected fragments were present but represent, in fact, a small fraction of the purified supercoil molecules. Due to the peculiar construction (heterogeneous ends) of the recombinants, some loss of HSV information was expected; nevertheless, the precise loss has yet to be determined. Some of the recombinants retain HSV information as evidenced by hybridization and cleavage with SmaI (which does not cleave SV40). The cloning of HSV-1 (KOS) into λ1059 has produced some 63 plaque isolates, each reputedly containing >7Kb HSV insert. Of 48 plaques tested, 44 hybridized with HSV-1 ^{32}P-DNA. These recombinants are being mapped and some have already been found to contain various contiguous EcoR1/Hind III fragments of HSV-1 (KOS) DNA.

Significance to Biomedical Research and the Program of the Institute:

The relevance of these studies rely on the medical importance of HSV. HSV is a ubiquitous human pathogen and possesses the ability to induce biochemical, neoplastic and morphological transformation of cells in culture. These studies will generate information and cloned HSV sequences which may serve as tools for potential screening of precancerous or cancerous tissues. Information generated on HSV replication may lead to preferred methods of treatment of HSV infections.

Proposed Course:

We intend to use the recombinants generated for studies of expression of isolated HSV genes in recipient cells (Xenopus or mammalian cells). The recombinants will also be the object of *in vitro* mutagenesis and reinsertion into infectious virus with the intent of obtaining novel mutants of HSV with lesions in Us.

Publications:

Colberg-Poley, A. M., Isom, H. C. and Rapp, F.: Involvement of an early human cytomegalovirus function in reactivation of quiescent herpes simplex virus type 2. *J. Virol*. 37: 1051-1059, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05213-01 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Expression of Eukaryotic Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael E. Dobson,	Guest Worker
OTHER:	George Khoury	Acting Chief, LMV, CIP, DCCP, NCI

COOPERATING UNITS (if any)

C. Gorman, LMB, NCI
B. Howard, LMB, NCI

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are investigating the effect of the 5' untranslated region of mRNAs on the stability of spliced and unspliced messages. We have ligated the 5' end of the HSV-TK gene extending from 15 nucleotides before the initiator AUG codon to ~550 nucleotides before the AUG into the 5' junction between the SV40 sequences and the bacterial chloramphenicol acetyltransferase (CAT) sequences in the recombinant plasmid pSV2-CAT. We have also removed a fragment from pSV2-CAT containing the SV40 t intron and splice sites, but not disturbing CAT sequences. These three recombinant plasmids have been used to transfect CV-1 cells and extracts from the cells analyzed for CAT activity. Cells transfected with the parent pSV2-CAT show considerable CAT activity as do those transfected with DNA lacking the t intron, while those transfected with the recombinant containing the S' end of the HTR gene show reduced activity.

PROJECT DESCRIPTION

Objectives:

To determine what effect the 5' untranslated region of mRNAs has on the stability and processing of spliced and unspliced messages.

Methods Employed:

Restriction enzyme analysis, recombinant DNA techniques, DNA transfection, in vitro CAT assay, thin layer chromatographs, RNA blotting technique, S1-nuclease analysis.

Major Findings:

pSV2-CAT can be used to transfect the CV-1 line of Monkey Kidney cells and Chloramphenicol acetyl transferase activity can be detected in extracts of these cells. When the t intron is removed no change in CAT activity was seen. However, when the S' end of the HTR gene was inserted between the SV40 promoter and the CAT gene in the pSV2-CAT recombinant, a significant decrease in CAT activity was seen.

Significance to Biomedical Research and the Program of the Institute

An understanding of the controls for mRNA processing and stability is fundamental to an understanding of genetic controls in general and can be extended to an understanding of the control of the various transformation genes in viral and non-viral systems.

Proposed Course:

We are constructing additional recombinant plasmids that lack the SV40 origin, promoter, and 5' ends but that contain the HSV-TK promoter, 5' ends and SV40 t intron and, a plasmid containing the SV40 origin, promoter and 5' ends the HSV-TK promoter and 5' ends but lacking the SV40 t intron region. These recombinants will be used to transfect CV-1 cells and we will analyze them for CAT activity. In addition, we will transfect COS cells, which contain a functional T antigen, with those recombinants containing the SV40 origin to analyze the CAT specific nuclear and cytoplasmic RNA species.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05214-01 LMV

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Elements Involved in the Initiation of Transcription

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter Gruss	Expert	LMV, NCI
OTHER:	Ravi Dhar	Visiting Scientist	LMV, NCI
	George Khoury	Acting Chief	LMV, NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Elucidation of the nucleotide signals, which serve as regulatory elements for the fundamental biochemical processes such as transcription and DNA replication, are crucial to an understanding of gene regulation. In this regard, small DNA viruses such as SV40 have been valuable model systems.

To the late side of the SV40 DNA replication origin are several sets of tandem repeated sequences, the largest of which is 72 base-pairs in length. The role of these sequences was examined through construction of deletion mutants of SV40. A mutant from which precisely one of the 72 pb bp repeated units was removed is viable upon transfection of monkey kidney cells with viral DNA. Extension of this deletion into the second repeated unit, however, leads to non-viability as recognized by the absence of early transcription and T-antigen production. These observations indicate that the 72 bp repeated sequences form an essential element in the early viral transcriptional promoter, and explain the inability of such a deleted genome to complement an early temperature-sensitive mutant of SV40, tsA, as well as the failure to replicate its DNA.

In a parallel experiment, it was found that the extended deletion mutant was also unable to complement a late temperature-sensitive mutant of SV40, tsB. This suggests that the extended mutant is also defective in DNA replication and/or late transcription.

PROJECT DESCRIPTION

Objectives:

This project is directed toward an analysis of the genetic elements required for the initiation of transcription. It should provide information about structure and function of eukaryotic promoters.

Methods Employed:

1. Construction of deletion mutants
2. Mapping of recombinant genomes
3. RNA analysis (see Project Z01 CP 055102-03 LMV)
4. Protein analysis " " "
5. Cloning in pBR322 and derivatives thereof.

Major Findings:

To differentially express the multiple cellular genetic programs, an intricate set of controls must regulate eukaryotic gene expression. One presumed target of these regulatory functions is the initiation of transcription. In this study, we examined the role of the largest SV40 repeat unit by deleting either one of the 72 bp copies (dl-2355) or most of both 72 bp repeats (dl-2356). Deletion of precisely one repeat unit results in a viable SV40 genome which is in agreement with earlier studies. This suggests that one set of the 72 bp repeats is sufficient to fulfill its biological function.

Extension of the single repeat (dl-2355) deletion into the second repeat resulted in a complete loss of early, as well as late, gene activity as evidenced by its failure to complementary (tsA28) or late (tsB4) temperature-sensitive mutants. Furthermore, direct biochemical analysis confirmed the biological assay, since neither early nor late cytoplasmic mRNA was detected after inoculation of dl-2356 DNA into monkey kidney cells. This is also reflected in the absence of early and late SV40 polypeptides (T-Ag and VP-1) as analyzed by immunoprecipitation. One interpretation of these data is that the 72 bp repeat is crucial to the initiation of early gene transcription. By analogy to prokaryotes, one would expect a certain amount of structural similarity among eukaryotic promoters required for the basic operation of the RNA polymerase II. Indeed, regions resembling genetic elements preceding prokaryotic 5' ends of mRNAs have been described. There is a TATAA sequence ("Goldberg-Hogness" or G-H box) preceding the cap site by approximately 25 nucleotides which closely resembles the sequence 10 nucleotides upstream from the prokaryotic mRNA start point (21). Although the structural similarity is evident, preliminary data suggest that the function of this genetic element differs. As opposed to prokaryotic Pribnow box, the G-H box seems to be dispensable for the *in vivo* expression of certain eukaryotic genes. One of its roles is apparently related to the positioning of the 5' end of eukaryotic mRNA.

One difficulty in searching for analogous sequences in other genes transcribed by polymerase II is our limited understanding of what elements constitute a eukaryotic promoter. Nevertheless, a number of similar repetitive sequences have been found at the 5' ends of certain genes.

Significance to Biomedical Research and the Program of the Institute:

The understanding of the molecular mechanisms leading to the initiation of transcription of eukaryotic genes is a prerequisite for the elucidation of controlling elements for activation of transformed genes and differentiation.

Proposed Course:

This project will continue with an emphasis on eukaryotic elements required for the initiation of transcription. The promoter-minus mutant dl-2356, cloned in pBR322 serves as acceptor for various DNA fragments harboring putative promoter elements. In our preliminary studies, we are inserting the 72, 73 bp repeats deriving from Moloney sarcoma virus into dl-2356.

Publications:

Gruss, P., Dhar, R., and Khoury, G.: Simian virus 40 repeated sequences as an element of the early promoter. *Proc. Natl. Acad. Sci. USA* 78: 943-947, 1980.

Gruss, P., and Khoury, G.: Gene transfer into mammalian cells: Use of viral vectors to investigate regulatory signals for the expression of eukaryotic genes. In Goebel, W., and Hofschneider, P.H., *Current Topics of Microbiology and Immunology*. Springer-Verlag, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05215-01 LMV

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Identification of Genetic Elements Involved in the Transcription of Selected Eukaryotic Genes Through the Use of Viral Vectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter Gruss	Expert	NCI, NIH
OTHER:	George Khoury	Acting Chief	NCI, NIH

COOPERATING UNITS (if any)

Drs. Nava Sarver and Peter Howley, Laboratory of Pathology, DCBD, NCI, NIH
Drs. Ronald Ellis, Thomas Shih and Edward Scolnick, Laboratory of Tumor Virus Genetics, DCCP, NCI

LAB/BRANCH

Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The complete rat preproinsulin gene I (rI_1) was cloned into an SV40 vector. Most of the late region of the viral vector (including the SV40 introns and all of the major splice junctions) was deleted and replaced by the entire rat insulin I gene. Analysis of the transcripts indicated that the rat preproinsulin gene nucleotide signals involved in RNA splicing and poly(A) addition were used. Examination of the 5' ends of the mRNAs showed several classes. This suggests that only a portion of the transcripts may be initiated faithfully. Significant quantities of a protein identified as rat proinsulin were synthesized, most of which is secreted. In an attempt to find a vector generating mRNA exclusively carrying authentic 5' end(s) (i.e. employing the cloned gene's promoter), a novel eukaryotic vector derived from the transforming region of bovine papillomavirus (BPV) was established and demonstrated to be highly effective for introducing foreign genes into animal cells. The foreign DNA is replicated as an episome, actively transcribed, and the transcripts are translated into an authentic gene product.

In another study, a cloned DNA fragment derived from Harvey murine sarcoma virus genome (Ha-MuSV) containing the p21 transforming sequences was cloned into the late region of an SV40 vector. The only significant class of mRNA transcribed from the recombinant is 930 nucleotides in length, unspliced, and initiated within Ha-MuSV sequences. This mRNA encodes large quantities of a transforming protein which is indistinguishable from the Ha-MuSV transforming protein p21. An mRNA with a 5' end similar to that transcribed from the recombinant has been identified in Ha-MuSV transformed mouse cells. In a claborative study, we have constructed a DNA hybrid mnolecule, BPV_{69T}-rI_1, containing the transforming region of BPV DNA and the rat preproinsulin gene I (rI_1) and used it to transform susceptible mouse cells. DNA hybridization analysis has demonstrated the presence of multiple unintegrated copies of hybrid DNA molecules with the BPV-1 DNA segment and the rI_1 gene covalently linked in selected transformed cell lines.

PROJECT DESCRIPTION

Objectives:

This project attempts to analyze genetic elements from various sources required for the faithful expression of selected eukaryotic genes. Viral vectors were employed allowing efficient introduction of these genes into the eukaryotic environment. The ultimate goal will be an understanding of regulatory mechanisms required for the selective expression of eukaryotic genes.

Methods Employed:

1. Construction of recombinant molecules: restriction endonuclease excision of DNA fragments; modification of the ends by addition of oligonucleotide "linkers;" ligation of DNA fragments; cloning of altered genomes by complementation with helper virus.

2. Mapping of the recombinant genomes. Analysis was performed with restriction endonucleases.

3. In order to determine the stable transcripts, the nuclease S-1 technique and a modification with Exo7 nuclease was used. In addition, "Northern" blots were hybridized with several nick-translated probes. Also, reverse transcription and subsequent sequence analysis was performed in order to determine the 5' end region of mRNAs.

4. Immunoprecipitations of proteins and electrophoresis in SDS-urea polyacrylamide gels was done to identify the proteins synthesized by the SV40-rat preproinsulin recombinant.

5. Cloning of DNA fragments in pBR322 and single-strand bacteriophage fd.

Major Findings:

SV40 Late Region Vectors. The insertion of recombinant DNA molecules within the late region of the SV40 genome is particularly advantageous in productive (lytic) infections of AGMK cells. Amplification of the templates in the presence of a functional T-antigen supplied by the recombinant molecule leads to a high copy number per cell. Subsequent to the viral DNA replication, the late SV40 promoters are particularly active and can lead to the expression of large numbers of transcripts from the recombinant region of these molecules. The result of such infections is in part the production of abundant RNA and protein molecules specific for the recombinant portion of the genome. A disadvantage to such reconstructed molecules is the potential use of the SV40 late promoter, in addition to a putative recombinant promoter.

In our initial studies, the complete rat preproinsulin gene I has been cloned into an SV40 vector. The initiation of stable transcripts of the insulin insert is as efficient as the production of late SV40 mRNA. Analysis of these transcripts indicated that the rat preproinsulin gene nucleotide signals involved in RNA splicing and poly(A) addition are used. The generation of a class of mRNA molecules similar in size to authentic rat insulinoma mRNA species, suggests that the initiation signal of the insulin insert is also functional. In the β cells of the pancreas, the primary translation product is a preprohormone (preproinsulin) which is processed stepwise into proinsulin and finally to mature insulin. The processing of preproinsulin to proinsulin involves the removal of the amino terminal 24 amino acids of the preprohormone. In monkey kidney cells (the permissive host for SV40), only the first processing step occurs which yields large quantities of proinsulin. The fact that most of the proinsulin accumulates in the tissue culture medium indicates that this protein is secreted. The maturation from proinsulin to insulin, however, has not been observed in monkey kidney cells. This processing step presumably requires the eukaryotic background of the highly differentiated pancreatic β cells.

A second study was directed at the expression of the Harvey murine sarcoma virus (Ha-MuSV) gene coding for the transforming protein p21. Ha-MuSV, a replication-defective retrovirus, originally was isolated by passage of Moloney murine leukemia virus (Mo-MuLV) in rats. The Ha-MuSV genome recently has been molecularly cloned and its DNA physically characterized.

We selected a 1.29 kilobase pair (kb) fragment, totally comprising the 5' region nonhomologous to rat 30 S RNA, for insertion into the late region of an SV40 vector. The data indicate that upon transmission to African green monkey kidney (AGMK) cells, this subgenomic fragment is stably expressed as a 0.93 kb mRNA derived entirely from the inserted DNA fragment and gives rise to large quantities of a 21K polypeptide. The p21 produced in AGMK cells is indistinquishable from the Ha-MuSV transforming protein.

A remarkable feature of RNA transcripts from the recombinant molecules was the presence of a major class of RNA containing discrete 5' termini mapping within the Ha-MuSV portion of the recombinant molecules. One interpretation of this finding is that a strong promoter for the $\mathrm{HaMu}_{\mathrm{src}}$ RNA exists just upstream from its translational initiation site. This expression may be independent of the distant LTR which maps 0.8-1.4 kb upstream, but in these molecules, may depend on the presence of the SV40 72 bp repeats. Since a similar, if not identical, transcriptional start site within the Ha-MuSV genome has been identified in Ha-MuSV-transformed mouse cells (in addition to the major RNA probably initiated in the LTR), it is very likely that an as yet unmapped promoter element directly precedes the 5' end sequences of the gene encoding p21.

A third study involves the use of bovine papilloma virus as a eukaryotic cloning vector. It has recently been demonstrated that the bovine papilloma virus can transform mouse cells while remaining in an unintegrated, episomal state. This suggested a potential to use BPV as a cloning vector which not only has the advantage of (a) containing its own selectable marker (viz. cell transformation), but also (b) a potential means for recovering the DNA of interest from the transformed cell by selecting the episomal fraction of DNA. Additional advantages of BPV as a cloning vector include the absence of a size restriction for encapsulation and the general amplification of the vector in between 20 and 200 copies per transformed cell. In our recent studies, it was demonstrated that the 69% transforming fragment of BPV could serve as an effective cloning vehicle for the rat I preproinsulin genes. S1 nuclease, in conjunction with Exonuclease VII analysis, revealed the presence of a correctly spliced body and a 5' terminus of preproinsulin transcripts similar or identical in its electrophoretic mobility to that of mRNA produced in rat insulinoma cells. Significant levels of a protein immunoreactive with anti-insulin serum were detected by radioimmunoassay in the culture medium of transformed cells. Immunoprecipitation analysis in conjunction with competitive binding to bovine proinsulin established the identity of the protein as that of rat proinsulin. These studies indicated that transcription was initiated within the rat preproinsulin portion of the molecule, presumably utilizing the preproinsulin promoter signals. This should be of advantage in future studies directed at the analysis of eukaryotic promoters. The production and secretion of rat proinsulin into the tissue culture medium suggested that this viral cell system may also serve as the source of protein encoded by recombinant molecules in eukaryotic cells. As such, the polypeptides produced by such a system have the potential advantage of undergoing eukaryotic post-translational modifications.

Significance to Biomedical Research and the Program of the Institute:

It is anticipated that the genetic elements which regulate the orderly and programmed expression of eukaryotic genes will provide an insight into those sequences which are aberrated in disease states such as neoplasia.

Proposed Course:

These projects will continue with two major aims:

1. Identification of the preproinsulin promoter and the promoter for the p21 src gene by further deletion mapping. Specifically, an insight into the interplay between the LTR promoter flanking the p21 gene and the putative internal p21 gene promoter should provide valuable information concerning the regulation of the p21 gene in transformed cells. This project includes the transcriptional analysis of the p21 related endogenous genes.

2. Vectors will be developed allowing the stable introduction of inducible genes (e.g. preproinsulin, rat growth hormone gene, rat prolactin gene) into their natural cellular environment. This project aims at the identification of genetic signals required for the induction or repression of the above mentioned genes.

Publications:

Gruss, P. and Khoury, G.: Expression of SV40 - rat preproinsulin recombinants in monkey kidney cells: Use of preproinsulin RNA processing signals. Proc. Natl. Acad. Sci., USA 78: 133-137.

Sarver, N., Gruss, P., Law, M.-F., Khoury, G., and Howley, : Bovine papilloma viral DNA - a novel eukaryotic cloning vector. J. Molec. Cellular Biol., in press, 1981.

Gruss, P. Ellis, R. W., Shih, T.Y., Koenig, M., Scolnick, E. M., and Khoury, G.: SV40 recombinant molecules express the p21 transforming protein of Harvey murine sarcoma virus from an unspliced RNA. Nature, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05216-01 LMV

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

DNA Sequence of Important Tumor Virus Genetic Elements

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ravi Dhar	Visiting Scientist	NCI, NIH
OTHER:	George Khoury	Acting Chief	NCI, NIH

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The DNA sequence of the transforming gene encoding the Harvey p21 gene product has been obtained. No RNA splice sites have been found within the coding sequence. We have localized and sequenced the putative RNA polymerase II and III initiation sites in this genetic segment.

PROJECT DESCRIPTION

Objectives:

1. A determination of the nucleotide sequence of p21 src gene coding for the polypeptide involved in transformation by Harvey sarcoma virus.

2. An analysis of the structure and localization of the regulatory regions of the Ha-src gene, such as promoters involved in transcription, polyadenylation and RNA splice sites.

Methods Employed:

5' and 3' end labeling, restriction endonuclease cleavage, polyacrylamide gel electrophoresis sequencing by 1) the chemical method of Maxim and Gilbert, and 2) an enzymatic method of Seif *et al.*, *Nucl. Acid Res.* 8: 2225, 1980.

Major Findings:

The Harvey strain of murine sarcoma virus encodes a p21 src protein which is required for transformation. p21 is among a class of normal cellular proteins whose genetic information has been incorporated into a transforming retrovirus. The nucleic sequence of Ha-MSV is composed of two distinct DNA sequences. The p21 coding sequence is flanked on both sides by a non-transforming retrovirus-like sequence cell called 30 S RNA. These sequences are flanked by murine long terminal repeated fragments (the sequence of which has been published [Dhar *et al.*, *Proc. Natl. Acad. Sci. USA* 77: 3937, 1980]).

We have obtained a DNA sequence of the region coding for p21 src sequences. This region of the genome can code in only one reading frame. There are multiple translational termination signals in other two frames. The DNA sequence preceding the translation initiation codon has two transcriptional promoters within a stretch of 100 nucleotides, one for RNA polymerase II and the other for RNA polymerase III. The nucleotide sequence known to be involved in RNA splicing is not present within the coding sequence (Seif *et al.* *Nucl. Acid. Res.* 6: 3387, 1979), thus apparently precluding the possibility of a second reading frame.

Under investigation at present are the endogenous sarc genes, we hope to determine if the cellular p21 is identical with viral p21 src. We are also in the process of locating the origins of RNA polymerase II and III sequences to determine whether they are present in the endogenous sequences or whether they are derived from 30 S RNA. This sequence will give an insight into the nature of recombination involved with 30S RNA (the nontransforming defective retrovirus).

Significance to Biomedical Research and the Program of the Institute:

The amino acid sequences of the src gene will help in elucidating the sequence which may be involved in its transformation activity and how its expression is regulated in cells.

Proposed Course:

1. Structure of the endogenus sarc genes.
2. Nature of recombination between MSV, 30tRNA and the endogenus sequences.
3. Two comparisons of a computer analyses of different transforming genes with that of the Harvey virus transforming gene, so that functions could be ascribed to different segments of the gene.

Publications:

Dhar, R., Chanock, R. M., and Lai, C.-J.: Non-viral oligonucleotides at the 5'-terminus of cytoplasmic influenza viral mRNA deduced from cloned complete genomic sequences. Cell 21: 495-500, 1980.

Dhar, R., McCLements, W. L., Enquist, L. S., and Vande Woude, G. F.: Nucleotide sequence of integrated Moloney sarcoma provirus long terminal repeats and their host and viral junctions. Proc. Natl. Acad. Sci. USA 77: 3937-3941, 1980.

Lai, C.-J., Markoff, L.J., Sveda, M., Dhar, R., and Chanock, R. M.: DNA sequence derived from genomic and mRNA species that code for the hemagglutinin and neuraminidase of influenza A virus: Structure and variation in influenza virus. In Laver G. and Air G. (Eds). Development in Cell Biology 5: Elsevier-North Holland, 1980, p. 115-124.

Seif, I., Khoury, G., and Dhar, R.: A rapid enzymatic DNA sequencing technique: Determination of sequence alterations in early Simian virus 40 temperature sensitive and deletion mutants. Nucl. Acid Res. 8: 2225-2240, 1980.

Lai, C.-J., Markoff, L., Sveda, M. M., Lamb, R., Dhar, R., and Chanock, R. M.: Genetic vartiation of influenza viruses as studied by recombinant DNA techniques. N. Y. Acad. Sci. 1980, in press.

McClements, W. R., Dhar, R., Blair, D. G., Enquist, L. W., Oskarsson, M., and Vande Woude, G. F.: The terminal repeats of integrated Moloney sarcoma provirus are like bacterial insertion sequences (IS) element. Cold Spring Harbor Symp. Quant. Biol. 45. 1980, in press.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05217-01 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Regulation of SV40 Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Shigeko Nomura	Microbiologist	LMV, NCI
OTHER:	Gilbert Jay	Visiting Scientist	LMV, NCI
	George Khoury	Acting Chief	LMV, NCI

COOPERATING UNITS (if any)

Dr. Carl Anderson, Brookhaven National Laboratory, New York

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the SV40 lytic infection of African green monkey kidney cells, SV40 agnogene, a sequence within the proximal late genome region, is expressed. A 7900 Mr (61 amino acid) polypeptide (the agnoprotein) was detected. The identity of this protein has been confirmed by comparing its amino-terminal sequence with that predicted from the SV40 nucleotide sequence. The agnoprotein accumulated late in the lytic cycle and had a half-life of approximately 2 hours. The highly basic nature of the protein and its affinity for both single- and double-stranded DNA suggested a possible regulatory role of this protein in nucleoprotein interactions.

BEST DOCUMENT AVAILABLE

PROJECT DESCRIPTION

Objectives:

To study the expression of the SV40 agnogene and the function of its product.

Methods Employed:

The detection and characterization of the SV40 agnoprotein were carried out on ^{14}C-arginine or leucine labeled cell extracts from SV40-infected African green monkey kidney cells by DNA cellulose chromatography, SDS polyacrylamide gel electrophoresis, immunoprecipitation and amino terminal sequence analysis. Analysis of viral transcripts selected by oligo (dT)-cellulose chromatography and by hybridization to DNA-containing filters was performed by in vitro translation and Northern blotting followed by hybridization with ^{32}P-labeled total SV40 DNA or the specific DNA segment isolated by restriction endonuclease digestion.

Major Findings:

1. The wild-type SV40 agnogene encodes a novel 7900 Mr (61 amino acid) protein. This protein (the agnoprotein) is absent from mock-infected cells, from cells infected by a mutant which contains a deletion over the entire region of the agnogene and from a 2 nucleotide insertion mutant which shifts the reading frame for this protein.

2. Amino terminal sequence analysis through the first 16 Edman cycles confirms the identity of the agnogene product and also indicates that the initiating methionine is quantitatively removed, leaving valine as the N-terminal amino acid. The absence of a methionine residue from the agnoprotein may account in part for previous failure to detect this protien.

3. Agnoprotein accumulates late in the lytic cycle and decays with a t1/2 of approximatley 2 hours. None of the available SV40 antisera lead to the immunoprecipitation of the agnoprotein.

4. The agnoprotein is highly basic (arginine + lysine = 25%), and it homogeneously and quantitatively binds both single- and double-stranded DNA.

5. The agnogene is not essential for virus growth. In a tissue culture system, a deletion mutant in the agnogene region appears to affect the production of late SV40 mRNAs, presumably by leading to a conformational change of the late RNA transcripts.

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Significance to Biomedical Research and the Program of the Institute:

Studies of SV40 gene products will hopefully provide an approach to the understanding of regulation of viral and host cell gene expression.

Proposed Course:

In order to understand possible roles of the agnoprotein in transcription, processing and attenuation of the late SV40 mRNA in both lytic and abortive infection, and in the assembly of virions, the following investigations are in progress:

1. Identification of RNA species which encode the agnoprotein.
2. Determination of the specific DNA binding site of the agnoprotein.
3. Localization of agnoprotein in the infected cell.
4. Evaluation of possible regulatory functions of agnoprotein on structural protein synthesis and virus maturation by comparing infections with the wild-type SV40 and its deletion or insertion mutants.

Publications:

Jay, G., Nomura, S., Anderson, C. W., and Khoury, G.: Identification of the SV40 agnogene product: A DNA binding protein. Nature, 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05218-01 LMV

PERIOD COVERED
October 1, 1980, through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Regulation of RNA Tumor Virus Gene Expression in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Shigeko Nomura	Microbiologist	LMV, NCI

COOPERATING UNITS (if any)
C. F. T. Mattern Medical Director LPD, NIAID
R. H. L. Pang, Genex Corporation, Rockville, Maryland

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To investigate the molecular mechanisms for malignant transformation, we are studying the expression of the transformation-specific protein p53 in MSV-transformed S+L- mouse cells and their flat revertants.

PROJECT DESCRIPTION

Objectives:

To investigate the function of the transformation-specific protein p53 and viral proteins in cell transformation and reversion.

Methods Employed:

Proteins were detected by immunoprecipitation using monoclonal and conventional antibodies.

Major Findings:

Preliminary observations indicate that normal parental, MSV-transformed S+L- and its flat revertant cells express varying amounts of p53. Detailed experiments are under way.

Significance to Biomedical Research and the Program of the Institute:

Studies of the cellular and viral functions involved in transformation and reversion will facilitate the understanding of molecular basis for viral oncogenesis and the nature of cellular regulatory control in mammalian cells.

Proposed Course:

Quantitative and qualitative analysis of p53 present in normal, MSV-transformed S+L- and revertant cells will be continued. To study the localization and arrangement of viral sequences in revertant cells, we will generate a restriction map of unintegrated m3-MSV (a Moloney murine sarcoma virus strain used to produce the S+L- cell line employed in these experiments) genome and clone the m3-MSV DNA molecule in a bacteriophage vector.

Publications:

Nomura, S., Daniel, W. A., Fernandez, J. A., Pang, R. H. L., and Mattern, C. F. T.: Morphologic changes in the rabbit SIRC cell line induced by simian sarcoma-associated virus. Virology 106: 395-400, 1980.

Fischinger, P. J., Blevins, C. S., Frankel, A. E., Tuttle-Fuller, N., Haapala, D. K., Nomura, S., and Robey, W. G.: Biological, immunological and molecular properties of revertants of cat cells transformed by murine sarcoma virus. Cancer Res. In press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05219-01 LMV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Construction of a General Vector for the Efficient Expression of Mammalian Proteins in Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gilbert Jay	Visiting Scientist	LMV, NCI
	George Khoury	Acting Chief	LMV, NCI

COOPERATING UNITS (if any)
Ernest Jay, Professor, Department of Chemistry, University of New Brunswick, Fredericton, N.B., Canada

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to investigate the regulatory elements required for recognition by prokaryotic ribosomes during the process of initiation of protein synthesis. We have generated a plasmid vector containing a synthetic ribosome binding site which will assure the efficient expression of mammalian proteins in Escherichia coli.

PROJECT DESCRIPTION

Objectives:

Use of a synthetic prokaryotic ribosome binding site for the efficient expression of SV40 t-antigen in bacteria.

Methods Employed:

Recombinant DNA techniques carried out under conditions specified by the NIH Guidelines were used in these studies. Nucleic acids were detected by combinations of hybridization, gel electrophoresis and electron microscopy. Proteins were analyzed by immunological methods, in combination with polyacrylamide gel electrophoresis.

Major Findings:

We have constructed a general plasmid vector carrying a chemically synthesized prokaryotic ribosome binding site which will assure the efficient expression of eukaryotic proteins in *E. coli*. In addition to the regulatory signals necessary for ribosome recognition, the synthetic segment contains, at one end, a Pst I cleavage site which will direct its insertion to pBR322 DNA and, at the other end, a Hind III site to facilitate attachment of the passenger eukaryotic gene. Using SV40 tumor (t) antigen as a model system, we have ligated the SV40 DNA fragment containing the entire t antigen gene in tandem with the synthetic ribosome binding site to pBR322 DNA at the Pst I site, which lies within the coding sequence of the β-lactamase gene. Initiation of transcription at the β - lactimase promoter would produce a chimeric messenger RNA with the synthetic ribosome binding signals and the SV40 sequence flanked by β-lactamase coding sequences. The utilization of the synthetic regulatory signals for the initiation of translation is demonstrated by the efficient synthesis in bacterial transformants of authentic SV40 t antigen. Excision of the entire SV40 insert by Hind III from those clones that have retained intact Hind III sites at the junction between the ribosome binding site and the SV40 sequence would allow insertion of other heterologous DNAs using Hind III linkers.

Significance to Biomedical Research and the Program of the Institute:

One of the main goals of recombinant DNA research is to produce significant quantities of medically and agriculturally important proteins whose genes have been cloned on bacterial plasmids.

With the premise that messenger RNAs transcribed in *E. coli* from cloned eukaryotic DNA inserts do not possess the necessary regulatory signals for recognition by prokaryotic ribosomes, we have constructed a general plasmid vector carrying a chemically synthesized prokaryotic ribosome binding site which will assure the efficient expression of eukaryotic proteins in *E. coli*.

This approach can be used for the expression of any eukaryotic protein whose RNA transcripts do not require post-transcriptional modifications specific to eukaryotic cells, or whose complementary DNA has been obtained by reverse transcription of its messenger RNA.

Publications:

Kaempfer, R., and Jay, G.: Binding of messenger RNA in initiation of prokaryotic translation. In Moldave, K. and Grosman, L. (Eds.): Methods in Enzymol. LX: Nucleic Acids and Protein Synthesis (Part H). pp 322-343, 1979.

Jay, E., Seth, A. K., and Jay, G.: Specific binding of a chemically synthesized prokaryotic ribosome recognition site: prospect for molecular cloning and expression of eukaryotic genes. J. Biol. Chem. 255: 3809-3812, 1980.

Jay, G., Khoury, G., Seth, A. K., and Jay, E.: Construction of a general vector for the efficient expression of mammalian proteins in bacteria: use of a synthetic ribosome binding site. Proc. Natl. Acad. Sci. USA, 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05220-01 LMV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Structure and Function of Cell Surface

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gilbert Jay	Visiting Scientist	LMV, NCI
	George Khoury	Acting Chief	LMV, NCI
OTHER:	David Cosman	Visiting Fellow	LMV, NCI
	Shigeko Nomura	Microbiologist	LMV, NCI

COOPERATING UNITS (if any)
L. J. Old, Vice President and Associate Director, Memorial Sloan-Kettering Cancer Center, New York, New York.

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to understand the molecular organization and expression of the mouse H-2 and Lyt surface antigens. We have been studying the histocompatibility H-2 antigens by molecular cloning technologies and the T-cell specific Lyt antigens with the use of monoclonal antibodies.

PHS-6040
(Rev. 2-81)

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PROJECT DESCRIPTION

Objectives:

1. Studies on the organization and expression of the genes coding for the histocompatibility H-2 antigens by molecular cloning technologies.
2. Studies on the molecular structure of the mouse Lyt-2 antigen by the use of monoclonal antibodies.

Methods Employed

Recombinant DNA techniques carried out under conditions specified by the NIH Guidelines were used in these studies. Nucleic acids were detected by combinations of hybridization, gel electrophoresis and electron microscopy. Proteins were analyzed by immunological methods, in combination with polyacrylamide gel electrophoresis.

Major Findings:

1. We have isolated from mouse DNA libraries, both genomic and cDNA clones which code for the mouse H-2 histocompatibility antigens. Identity of these clones was shown by direct DNA sequence analysis, and comparison of the deduced sequence with the known amino acid sequence for the H-2 antigens. The availability of such clones will allow the study of the genetic complexity, polymorphism and expression of the H-2 genes.

2. Thymocytes from BALB/c mice were radiolabeled in culture, and the resulting cell extract was subjected to immunoprecipitation with a monoclonal anti-Lyt-2.2 antibody. Analysis of the immunoprecipitates on SDS-polyacrylamide gels in the presence of a reducing agent revealed three components that were specifically recognized by the monoclonal antibody; they have subunit molecular weights of 35,000, 30,000, and 28,000. By combining with polyacrylamide gel analysis in the absence of reducing agents, it was shown that there are two forms of the Lyt-2.2 antigen, each consisting of one molecule of the 28,000 M_r subunit covalently associated through disulfide bonds with either one 35,000 M_r subunit or one 30,000 M_r subunit. These two molecular structures are present in about equimolar ratios.

Significance to Biomedical Research and the Program of the Institute:

1. The H-2K and H-2D loci of the mouse histocompatibility complex code for structurally-related membrane glycoproteins which determine compatibility in tissue or organ transplantation. The complexity in allograft rejection is the result of their genetic polymorphism; that is, the existence of multiple alleles at each individual H-2 locus. A conservative estimate indicates that there are at least 56 alleles at the H-2K locus and 45 alleles at the H-2D locus, thus allowing for some 2,500 different combinations between these two loci alone. Since there is a high degree of

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heterozygosity at these loci (that is, the presence of different alleles at the two genes in the homologous chromosomes), most of these combinations probably do exist in the mouse population. In addition, we have come to recognize that these H-2 antigens also play an important role in the immune response to foreign antigens. Specifically, the H-2K and H-2D loci are known to restrict effector lymphocyte specificity in such a way that maturing lymphocytes learn to recognize foreign antigens associated with the H-2K and H-2D molecules of the sensitizing cell. Success in cloning these genes will allow us to study the genetic complexity, polymorphism, and gene expression of the H-2 histocompatibility antigens.

2. Three systems of surface antigens, Lyt-1, Lyt-2 and Lyt-3, characterize cells of thymic derivation in the mouse. As no other cell type has been found to express these components, Lyt antigens have been particularly useful as T-cell markers. Considerable interest in Lyt-2,3 antigens has been generated by the recent finding that conventional and monoclonal antibodies to these determinants in the absence of added complement can block T-cell cytotoxicity. It has been suggested that molecules bearing Lyt-2,3 determinants were in close spacial proximity to antigen receptors on T-cells, either on unrelated adjacent molecular structures or as an integral component of the receptor. The finding that Lyt-2,3 genes are tightly linked to structural genes for κ light chains adds support to the speculation that Lyt-2,3 components may be involved in the construction of one class of T-cell receptors, in which κ chains and Lyt-2,3 chains would comprise the subunits of a functional recognition unit. Biochemical studies may provide an insight into the close linkage of the genes coding for Lyt-2 and -3 and kappa light chains and the meaning of specific locking of cytotoxic T-cells by Lyt-2,3 antibody.

Proposed Course:

We are attempting to further define the molecular complexities of these surface antigens at both the structural and functional levels.

Publications:

Jay, G., Ferrini, U., Robinson, E. A., Khoury, G., and Appella, E.: Cell-free synthesis of mouse histocompatibility (H-2) antigens. Proc. Natl. Acad. Sci. USA 76: 6562-6566, 1979.

Jay, G., Palladino, M., Khoury, G., and Old, L. J.: The mouse Lyt-2 surface antigen: evidence for a covalently-associated heterodimeric structure. Proc. Natl. Acad. Sci. USA, 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05221-01 LMV

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

lerpes Simplex Virus Genomic Arrangement in DNA Transfected and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Bear	Staff Fellow	LMV, NCI
OTHER:	Lynn W. Enquist	Research Scientist (PHS)	LMV, NCI
	Anamaris M. Colberg-Poley	Postdoctoral Fellow	LMV, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genetic control elements in herpes simplex virus type 1 (HSV-1) are investigated. Deletions occurring in a 9.5 base pair EcoR1 defective DNA fragment co-transfected with the HSV-1 tk gene in mouse Ltk⁻ cells are analyzed. Tumors generated in vivo from an inoculation of cells transformed in vitro by HSV-1 were negative for retention of virus-specific sequences.

PHS-6040
(Rev. 2-81)

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PROJECT DESCRIPTION

Objectives:

The objective of this work is to study the arrangement of the herpes simplex virus genome and of specific cloned segments of the viral genome in DNA transfected cells and transformed tumor cells by a) identifying and locating specific HSV-1 sequences in cells from rat tumors generated by an inoculation of in vitro HSV-1 transformed syngeneic cells and b) identifying control regions in HSV-1 DNA present in defective DNA (dDNA) of HSV-1. This dDNA is found as a repeated sequence in defective viral particles and most probably contains active replication and packaging sites. It has been classified as a member of the major class group I dDNA (Denniston, et al., 1981). In addition, it is known to carry two promoters for immediate early messenger RNA synthesis (Watson, 1981). Work in this laboratory has also suggested the presence in a 9.5 kb dDNA fragment of the terminal "a" sequence which may be active in HSV recombination (Z01 CP 05102-03 LMV).

Methods Employed:

Two cell lines were received (J. McNab, 1974, 1975) and maintained in vitro: a) a tumor cell line generated by an inoculation into rats of syngeneic cells transformed in vitro by sheared HSV-1 DNA (McNab, 1974, 1975) and b) syngeneic normal rat fibroblasts. Both total cellular DNA and extra chromosomal DNA were isolated, digested with the restriction endonuclease EcoR1, electrophoresed in 0.7% agarose gels and transferred to nitrocellulose membranes by the technique of Southern. Hybridization of this cellular DNA was performed using specific radiolabeled sequences of HSV-1.

A 9.5 kb EcoR1 dDNA fragment (major class group I), pBR322tk plasmid DNA and a phage vector (λWES.B) carrying the 9.5 kb EcoR1 dDNA fragment (λWES::12-7) have been described previously (Z01 CP 04882-06 LMV and Z01 CP 05102-03 LMV).

A hybrid pBR325 plasmid will be constructed by methylation of EcoR1 sites in the Bam H1 tk fragment and insertion into the Bam H1 site of pBR325. This plasmid will be digested with EcoR1 and the 9.5 kb EcoR1 dDNA fragment will be ligated into the plasmid EcoRI site. A plasmid carrying the two inserts will be identified after transfection of DNA into E. coli and selection for $\text{Amp}^R\text{Tet}^S\text{Cam}^S$ colonies. Restriction endonuclease analysis of this plasmid DNA will confirm the presence of both inserts. A lamda coliphage carrying both inserts will be generated by a recombination between λWES::12-7 (immλ) and λ imm) 434::tk. The phage will be selected from CsCl gradient fractions for the production of turbid plaques on YML(λ). Restriction endonuclease analysis of the phage DNA will confirm the presence of both inserts. DNA from tk$^+$ transformant colonies will be analyzed by restriction enzyme digestion, Southern transfer, and hybridization to specific HSV DNA.

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Major Findings:

1) Cells transformed *in vitro* by sheared HSV-1 DNA contained HSV-1 sequences which could be retrieved by superinfection with a temperature-sensitive HSV-1 mutant (McNab, personal communication). These sequences were localized in areas of the HSV genome equivalent to 0.6-1.0 map units. Utilizing an HSV-1 probe specific for sequences in TR_S and the right-hand portion of U_S, we were unable to detect any HSV-1 sequences in the rat tumor cell DNA restricted with *EcoR1*. Clearly, although HSV-1 sequences may be retained by cells transformed *in vitro*, these portions of the genome do not appear necessary for tumor growth *in vivo*. As indicated in the following section it may be that HSV-1 DNA undergoes rearrangements and deletions in eukaryotic cells preventing detection of viral DNA in transformed lines.

Restriction enzyme analysis of genomic DNA from tk$^+$ transformed mouse L cells indicated extensive editing of this 9.5 kb *Eco*R1 HSV-1 dDNA fragment. To determine if this editing was due to the segregation of the non-selectable dDNA in the co-transfected cells or was due to rearrangement induced either by the presence of poison (transcription or replication) sequences encoded in this fragment or by cellular mechanisms, hybrid plasmid and phage vectors each carrying the HSV tk gene and the 9.5 kb *Eco*R1 dDNA fragment are being constructed. Analysis of eight tk$^+$ transformant colonies to identify rearrangement of HSV-1 DNA is proceeding.

Significance to Biomedical Research and the Program of the Institute:

Herpes virus has been shown to transform cells *in vitro*, but Southern analyses of these cells fails to detect resident segments of viral DNA. The loss of DNA sequences may suggest a hit and run mechanism. To test this hypothesis, we are establishing a control system for examining the loss of HSV-1 DNA from eukaryotic cells. The 9.5 kb *Eco*R1 dDNA fragment, a repeated sequence in defective virus particles, contains many control sequences. This segment may direct its own rearrangement, or exhibit deletions that result from cellular control. An understanding of this region will contribute to the knowledge of the genetic recombination in eukaryotic cells. Such mechanisms are postulated to play a role in spontaneous neoplasms (Z01 CP 04882-06 LMV).

Proposed Course:

Study tk$^+$ transformed clones to identify those containing controlling elements co-transformed from HSV-dDNA; analyze the mechanism of HSV induced transformation and effects of specific HSV genetic segments on cellular processes.

Publications:

None.

SUMMARY REPORT

LABORATORY OF TUMOR VIRUS GENETICS

October 1, 1980 through September 30, 1981

The Laboratory of Tumor Virus Genetics has extended its studies on the origin, structure and function of viral transforming genes and their gene products. To understand the origin of the transforming genes, we have molecularly cloned segments of the src gene of Harvey and Kirsten sarcoma virus. Two bands with homology to the Harvey src gene have been identified in rat DNA digested by EcoRI endonuclease. We cloned each of these segments and have shown that one gene is colinear with the src gene of Harvey virus while one gene has introns. Each normal rat gene is capable of inducing malignant transformation of cells and codes for p21, the src gene product of Harvey virus. Our results prove unambiguously that activation of a normal cellular gene can cause malignant transformation. We are comparing the biochemical properties of the p21 coded by the viral and cellular src genes and our studies have already revealed some interesting differences between them. These studies may help to elucidate the biochemical properties of p21 which are most closely associated with transformation. Surprisingly, we have discovered that p21 genes represent a family of divergent genes in normal rat DNA. A unique hemopoietic stem cell line expresses one of these genes and the results suggest that p21 genes may play a role in normal hemopoietic stem cell physiology and differentiation. In addition, p21 has been shown to be a subunit of a membrane bound protein kinase and these results have provided a major insight into the elucidation of the metabolic pathway(s) involved in the control of cell growth.

In parallel studies, an *in vitro* system was developed to identify the promotors of transcription of src genes. Promotors for RNA polymerase II and surprisingly, RNA polymerase III, were clearly identified. This is quite unexpected since no mRNA with protein coding potential has previously been associated with RNA polymerase III activity. Since activation of cellular src genes has been shown to be capable of inducing transformation, these transcriptional control studies are quite important for elucidating natural control mechanisms and targets for activation of endogenous src genes by chemical and physical carcinogens.

In other studies on transcriptional control mechanisms, significant progress has been made in a hormonal system which we have been studying for several years. The mouse mammary tumor virus is a unique retrovirus in that the rate of MMTV RNA synthesis is regulated by glucocorticoid hormones. Earlier work had suggested that the recognition signals for glucocorticoid stimulation were a part of the genomic structure of MMTV. A full length DNA form of an endogenous MMTV has been molecularly cloned and retains the glucocorticoid regulatory signals. The molecular clone has been used to construct chimeric molecules containing the p21 src gene of Harvey virus and the LTR of MMTV. These chimeric molecules seem to be hormonally responsive to glucocorticoids. These results suggest that we have identified a key regulatory element in the mechanism of action of steroid hormones and this system will be actively pursued in the next few years. Since steroid hormones have a wide clinical use, these studies are of fundamental significance in future clinical applications.

In our studies on Friend virus induced erythroleukemia, we devised an assay to perform a genetic analysis of the leuk gene of spleen focus-forming virus. Using molecularly cloned SFFV in this assay, we have localized the leuk gene of this virus to the terminal 1500 bases of the viral DNA. This data provides further support of our previous observations that the env-related viral protein coded by this segment of the viral genome is a product of the leuk gene. We are now making mutants and performing sequence analysis on the gene. In other studies on the Friend virus complex, a novel mechanism of resistance to viral leukemogenesis has been identified. Certain strains of mice express a glycoprotein which competes with the viral gene product associated with the induction of leukemia. This is the first biochemical identification of a cellular gene product that confers resistance to viral leukemia. An analysis of the interactions between this host glycoprotein and viral coded gene products should provide a great deal of insight into the basic mechanisms of leukemogenesis.

Finally, we have devised a new system utilizing the Harvey sarcoma virus to construct a retrovirus vector which has been used to transmit a nononcogenic gene (HSV-TK). This is the first time retroviruses have been used as vectors for nontransforming genes; and this technique offers a new approach to introducing genes into hemopoietic cells and possibly, in the future, for genetic therapy for hemopoietic and other diseases in man.

In summary, the Laboratory of Tumor Virus Genetics has continued to make significant progress in understanding viral and cellular-induced carcinogenesis at a molecular level. In addition, the Laboratory has significantly broadened the directions of its research to include hemopoietic cell biology, transcriptional control, and new ways of introducing genes into cells.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04846-09 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Analysis of Viral Infection and Its Control at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. I. Gerwin	Chemist	LTVG NCI

COOPERATING UNITS (if any)

D. Lowy	Medical Officer	DCB, NCI
J. Levin	Chemist	LMG, NICHD
A. Rein	Frederick Cancer Research Center	

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are utilizing a non-conditional polymerase mutant of B-tropic MuLV to study the mechanism of the reverse transcriptase reaction. This mutant synthesizes partial copies of the natural template in vitro. These products are being characterized in order to determine the nature of the enzymatic blockage to completion of the transcription reaction. At the same time, we are attempting to clone this mutant molecularly in order to completely characterize the genetic change which has led to the structural alteration in the polymerase. In addition, other mutants of tropic MuLVs are being constructed. It is hoped that these various classes of mutants will enable us to correlate known genes and gene products with specific viral functions.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The goal of this project is an understanding at the molecular level of the life cycle of RNA tumor viruses, i.e., the expression of these viruses in cells infected both nonproductively and productively, as well as an understanding of the cellular control mechanisms applicable to these viruses.

The topics of present interest are:

(A) Analysis of the products of in vitro DNA synthesis by a mutant polymerase molecule. Further characterization of the mutant viral genome which codes for this protein.

(B) Generation and characterization of mutants of endogenous leukemia viruses.

Methods Employed:

(A) Enzymes are purified from disrupted virions and cells using affinity and ion-exchange chromatography and velocity gradients. Products of DNA synthesis by mutant and wild-type enzymes are compared by gel electrophoresis, DNA blotting techniques and filter hybridization. Intermediates are isolated and characterized by annealing to specific probes and by restriction enzyme digestion. Kinetics of enzymatic reactions are analyzed.

(B) A plasmid carrying a complete, infectious copy of an endogenous RNA tumor virus genome will be mutagenized. This will be accomplished in a specific fashion by introducing new, defined sequences at specific positions in the genome. This altered DNA will be used to infect bacteria. Desirable mutants will be selected and studied for infectivity, blockage at specific steps in the virion life-cycle and for their ability to complement or be complemented by other defined RNA tumor virus mutants or genome fragments.

Major Findings:

(A) The altered polymerase molecule present in the mutant virions begins to copy the native genome but is unable to complete this reaction. The change in the structure of the enzyme induced by the mutation has presumably eliminated a function required to synthesize a complete DNA copy. Thus, it appears that analysis of this mutant may define a partial reaction involved in reverse transcription.

(B) A defective leukemia virus isolated from a transplantable murine leukemia cell has been studied. Although the only viral protein synthesized by this mutant is an envelope glycoprotein, the genome has been shown to be full length. Thus far it differs from that of AKR in processing an altered sequence within the 5' "gag"-gene coding region.

Significance to Biomedical Research and the Program of the Institute:

(A) Further characterization of this polymerase mutant will increase our understanding of the mechanisms of RNA tumor virus replication. Identification of a sensitive portion of this reaction may reveal a phase of the viral life cycle which would provide a target for chemotherapy.

(B) There are few non-conditional mutants of mammalian RNA tumor viruses. The development of well-characterized mutants will provide important tools for analysis of infection by RNA tumor viruses both *in vivo* and *in vitro*. The availability of such mutants as recombinant DNA clones will allow precise mapping of the defect on the genome and provide information as to the functional significance of known genes. Such information would be of importance in understanding how these viruses produce cancer.

Proposed Course:

(A) Those intermediates which are formed in the abortive polymerization catalyzed by the mutant will be characterized to identify the point at which the polymerization reaction is blocked. The mutant genome will be molecularly cloned and then mapped and R-looped with wild-type virus to locate the defect on the viral genome.

(B) Mutants will be generated and analyzed to correlate functional blockages with structural defects.

Publications:

Bassin, R. H., Gerwin, B. I., Levin, J. G., Duran-Troise, G., Benjers, B. M., and Rein, A.: Macromolecular requirements for abrogation of Fv-1 restriction by murine leukemia viruses. *J. Virol.* 35: 287-297, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT**	**PROJECT NUMBER** Z01 CP 04848-09 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

RNA Tumor Viruses: Replication, Transformation and Inhibition in Cell Cultures

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. H. Bassin	Head, Viral Biochemistry Section	LTVG	NCI
OTHERS:	S.K. Ruscetti	Senior Staff Fellow	LTVG	NCI
	Z. Selinger	Visiting Scientist	LTVG	NCI

COOPERATING UNITS (if any)

A. R. Rein Frederick Cancer Research Center

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	**PROFESSIONAL:** 1.0	**OTHER:** 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

DBA/2 mice are resistant to induction of disease by Friend MuLV, a process which appears to require the formation of recombinant MCF viruses. This resistance appears to be due to the presence of an endogenous MCF-like glycoprotein on the surface of DBA/2 cells, which blocks MCF receptors on these cells. A $gag^{-}pol^{-}env^{+}$replication-defective MuLV was characterized and was used in experiments analyzing the resistance of DBA/2 cells to MCF. Treatment of DNA/2 cells, as well as cells producing MuLVs, with inhibitors of glycosylation reverses viral interference.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The primary objective of this effort during the past year has been an understanding of mechanisms of resistance to murine leukemia virus-induced disease. The experiments are aimed at elucidating the steps in the viral replication cycle that are potential sites of interruption.

Methods Employed:

(A) Murine leukemia viruses are quantitated by a variety of cell culture procedures including the S^+L^- focus assay, the XC test, and, when necessary, assays for cell transformation. Additional assay techniques are developed and characterized as needed, as are stable cell lines.

(B) Southern blotting techniques, nick translation of viral DNA, and restriction enzyme digests of viral DNA intermediates.

Major Findings:

A replication-defective MuLV, originally isolated in this laboratory from cultured AKR mouse lymphoma cells, has been characterized. It directs the synthesis of an ecotropic env protein, but no gag or pol proteins. Its genome contains a novel restriction endonuclease cleavage site near the beginning of the gag gene; this alteration may be responsible for the failure of this virus to synthesize gag or pol proteins. Cells containing this virus exhibit type-specific interference to ecotropic MSV pseudotypes; therefore, the env protein alone is sufficient to induce viral interference. This virus has been used as a reagent in the studies described below.

DBA/2 mice are resistant to disease induction by ecotropic Friend MuLV, even though this virus replicates well in these mice. In studies to explain this resistance, it was found that DBA/2 mice differed from susceptible mice in that recombinant (MCF) viruses could not be recovered from their spleens after injection of ecotropic Friend MuLV. In vitro tests showed that DBA/2 cells are specifically resistant to infection with MCF. These cells express an 80,000 dalton glycoprotein on their cell surface which is specifically precipitable by anti-MCF antiserum. These findings suggested the possibility that 1) MCF viruses may be involved in induction of disease by ecotropic Friend MuLV and 2) DBA/2 cells are resistant to infection with MCFs by a mechanism analogous to viral interference, i.e., an endogenous MCF-specific glycoprotein in DBA/2 cells blocks those cell surface receptors by which pathogenic MCFs could enter the cell.

This hypothesis was tested by two novel approaches. First, MCFs were grown in cells containing the replication-defective ecotropic MuLV described above. This defective MuLV was able to donate env (but no other viral products) to the MCFs. The resulting phenotypically mixed MCF particles were then found to be able to infect DBA/2 cells, thus confirming that the resistance of these cells to normal MCFs is specifically directed at the MCF env protein.

It has also been found that standard viral interference (i.e., the specific resistance of cells producing one MuLV to superinfection with a homologous virus) is specifically reversed by treating the cells with the glycosylation inhibitors 2-deoxyglucose and tunicamycin. When DBA/2 cells were treated with these anti-metabolites, they were found to become susceptible to infection by MCFs, thus confirming the analogy between the resistance of DNA/2 cells to MCFs and standard viral interference.

Significance to Biomedical Research and the Program of the Institute:

The analysis of the resistance of DBA/2 mice to disease induction by ecotropic Friend MuLV indicates that replication of recombinant MuLV in these mice is a necessary step in this disease process, and also serves as a model for one mechanism of resistance to virus-induced disease. In addition, new reagents and experimental approaches (i.e., a $gag^{-}pol^{-}env^{+}$ replication-defective MuLV and the reversal of viral interference by inhibitors of glycosylation) have been introduced which should prove very useful in future analyses of the viral replication cycle.

Proposed Course:

The experiments described above suggest that DBA/2 cells are nonpermissive for MCF, and that infection by MCF is necessary for disease induction by Friend MuLV. In addition, these experiments have demonstrated two ways of infecting DBA/2 cells with MCF *in vitro*: donation of an ecotropic env protein to MCF by phenotypic mixing and pretreatment of the cells with inhibitors of glycosylation. These findings can now be used to investigate the question of the cellular target for disease induction by MCF. Hematopoietic precusor cells will be removed from DBA/2 mice and fractionated *in vitro*. The different fractions will then be infected with MCF by one of the methods described above and re-injected into DBA/2 mice; that fraction which, when successfully infected, produces disease upon re-injection, will presumably contain the target cell type.

Publications:

Yang, W. K., Kiggans, J. O., Yang, D.-M., Ou, C.-Y., Tennant, R. W., Brown, A., and Bassin, R. H.: Synthesis and circularization of N- and B-tropic retroviral DNA in Fv-1 permissive and restrictive mouse cells. *Proc. Natl. Acad. Sci. USA* 77: 2994-2998, 1980.

Duran-Troise, G., Bassin, R. H., Wallace, B. F., and Rein, A.: Balb/3T3 cells chronically infected with N-tropic murine leukemia virus continue to express Fv-1^{b} restriction. *Virology* (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04858-09 LTVG

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Control of Growth and Differentiation of Fibroblastic and Hemopoietic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. M. Scolnick	Chief	LTVG	NCI
OTHER:	T. M. Shih	Research Chemist	LTVG	NCI
	D. Lowy	Medical Officer	DCB	NCI
	R. Ellis	Staff Fellow	LTVG	NCI
	D. Linemeyer	Staff Fellow	LTVG	NCI

COOPERATING UNITS:

C. M. Wei	Expert, Laboratory of Viral Carcinogenesis, NCI
H. Young	Frederick Cancer Research Center, Frederick, MD.
T. M. Dexter	Paterson Laboratories, Manchester, England
P. Spear	University of Chicago, Chicago, Illinois
M. Spector	Cornell University, Ithaca, New York
E. Racker	Cornell University, Ithaca, New York

COOPERATING UNITS (if any)

(see above)

LAB/BRANCH

Carcinogenesis Intramural Program

Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	3.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolism and enzymology of the transforming protein, p21, of Harvey and Kirsten sarcoma viruses have been investigated. A precursor polypeptide to the p21 has been detected and two processed product forms of the protein chased from the precursor. Tentative evidence has been obtained that the p21 is a subunit of a membrane associated phosphokinase. A unique hemopoietic precursor cell line has been identified which expresses high levels of an endogenous p21, suggesting that p21 metabolism plays a role in controlling proliferation of hemopoietic stem cells.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The goal of this project is to define the biochemical pathways involved in different forms of different cancers and to define the normal physiological roles for the p21 protein in cellular functions, especially hemopoietic cellular functions. The long-range goal is to refine assays for p21 so that they can be used in diagnosis of human diseases including cancer.

Methods Employed:

A. Pulse labeling of cells with ^{35}S-methionine and immunoprecipitation using Staphylococcus aureus protein A. Analysis of precipitates on polyacrylamide slab gels. Fluorographic enhancement of protein bands. Tryptic peptide analyses using two-dimensional thin layer plates.

B. Restriction enzyme analyses of cloned and amplified sarcoma virus DNA. Southern transfer blotting assays and ethidium bromide containing cesium chloride gradients.

C. In vitro culture of bone marrow cells. Prolonged growth in cell culture of hematopoietic stem cells and granulocyte and erythroid precursor cells. Soft agar assays and methylcellulose assays for these cells. Histological identification of various hematopoietic cells.

D. Biochemical assays for phosphorylation and dephosphorylation of proteins. Gel electrophoresis, charcoal extractions, butanol two-phase extractions, thin layer chromatography.

E. Column chromatography using ion-exchange, hydroxyapatite, and hydrophobic column chromatography. Analysis of peptides by two-dimensional thin layer chromatography.

F. Gene transfer studies using liposomes, calcium phosphate precipitation and protoplasts, gene cloning using a variety of bacterial vectors.

Major Findings:

A. The metabolism of p21 src protein has been studied in order to define which biochemical activities of p21 are associated with which structural forms of p21. A precursor of approximately 24,000 daltons has been identified which is processed to a 20,000 dalton form. A phosphorylated form of p21 is formed from the processed 20,000 dalton form of p21. The amino terminus of both the pro p21 and p21 are blocked. The precursor of p21 is made in the cytosol, not attached to membranes, while the processing occurs in association with membranes. Work is in progress to try to isolate the enzymes involved in processing and to separate large amounts of the different forms of p21.

B. A hemopoietic stem cell line, 416B, has been identified which expresses high levels of an endogenous p21. The levels of p21 are even higher than the levels detected in Harvey virus transformed cells. The results suggest a role for p21 in controlling decisions of proliferation vs differentiation of hemopoietic cells. Since Dr. Ellis, in my laboratory, has shown that there is a family of p21 genes, the tissue-specific expression of p21 in different specialized cells becomes an important problem.

C. A retrovirus vector system for gene transfer has been constructed using Harvey virus. This is the first demonstration of retroviruses as vectors for nononcogenic genes. The thymidine kinase gene of herpesvirus has been placed in a new retrovirus which carries both the src gene of Harvey virus and the tk gene of herpes-virus. This retrovirus can be transmitted to new cells with type C helper viruses.

Significance to Biomedical Research and the Program of the Institute:

One of the major problems is to be able to introduce specialized genes into pluripotent cells. The retrovirus vector system that we have devised allows us to introduce a variety of genes into hemopoietic stem cells and opens the possibility in the future for gene therapy of hemopoietic cells. The unique ability of retroviruses to propagate in hemopoietic cells coupled with this vector system allows a whole new range of approaches to hemopoietic gene transfer studies. The metabolism of the p21 src gene has led to the tentative identification of a membrane-associated phosphokinase of which p21 is a subunit. The realization that p21 is a regulatory subunit for a membrane kinase allows us to study how p21 causes cellular transformation.

Proposed Course:

We will purify to homogeneity the membrane kinase which p21 is associated with. We wish to determine the targets of this kinase and how p21 regulates the activity of the kinase. Antibodies to the other subunit of the kinase will be prepared, and we will attempt to identify the mRNA for this subunit with the eventual goal of cloning the gene for that subunit and locating its relationship to p21 genes in the genome of various vertebrate cells. We will molecularly clone our new retrovirus vector and begin to use it in a variety of gene transfer studies.

Publications:

Shih, T. Y., Papageorge, A. G., Stokes, P. E., Weeks, M. O. and Scolnick, E. M.: Guanine nucleotide-binding and autophosphorylating activities associated with the p21^{src} protein of Harvey murine sarcoma virus. Nature 287: 686-691, 1980.

Linemeyer, D. L., Ruscetti, S. K., Scolnick, E. M., Evans, L. H. and Duesberg, P. H.: Biological acitivity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA. Proc. Natl. Acad. Sci. 78: 1401-1405, 1981.

Scolnick, E. M.: Transformation by rat-derived oncogenic retroviruses. Microbiological Reviews 45: 1-8, 1981.

Scolnick, E. M., Weeks, M. O., Shih, T. Y., Ruscetti, S. L. and Dexter, T. M. Markedly elevated levels of an endogenous sarc protein in a hemopoietic precursor cell. Molecular and Cellular Biology 1: 66-74, 1981.

DeFeo, D., Gonda, M. A., Young, H. A., Chang, E. H., Lowy, D. R., Scolnick, E.M., and Ellis, R.: Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. in press.

Wei, Cha-Mer, Gibson, M., Spear, P., and Scolnick, E. M.: Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of Herpes simplex virus type I. J. Virol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04899-09 LTVG

PERIOD COVERED

October 1, 1980 through September 30, 1981.

TITLE OF PROJECT (80 characters or less)

Transforming Genes of Avian RNA Tumor Viruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. S. Papas	Head, Carcinogenesis Regulation	LTVG	NCI
OTHER:	J. A. Lautenberger	Expert	LTVG	NCI
	K. Rushlow	Guest Worker	LTVG	NCI
	K. P. Samuel	Visiting Fellow	LTVG	NCI
	P. N. Tsichlis	Expert	LTVG	NCI
	S. Aaronson	Chief	LCMB	NCI
	S. Tronick	Chemist	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)

J. Chirikjian	Biochemistry Dept., Georgetown University
P. Duesberg	Molecular Biology Dept., Univ. of Calif., Berkeley, Calif.
M. Baluda	Dept. of Pathology, UCLA, California
E. Canaani	Weizmann Institute, Israel

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 3.0 PROFESSIONAL: 2.0 OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have used recombinant DNA technology to isolate and characterize the avian myelocytomatosis (MC29) sequences which are essential in the transformation process. Integrated MC29 proviral DNA was isolated from a library of recombinant phage containing DNA from the MC29-transformed nonproducer quail cell line Q5. The cloned DNA was analyzed by Southern blotting of restriction endonuclease digests and by electron microscopic visualization of R-loops formed between the cloned DNA and MC29 or helper virus RNA. It was found that the 9.2 kb cloned DNA insert contains approximately 4 kb of viral sequences and 5.2 kb of quail cellular sequences. The viral sequences contain all of the MC29-specific sequences and 5' helper-related sequences, as well as part of the envelope region. The size of the cloned EcoRI fragment is the same as that of the major band in EcoRI-cleaved Q5 DNA that hybridizes to viral sequences. Transfection of the cloned DNA into NIH 3T3 cells revealed that the MC29-specific sequences are functional in that they induce foci of transformed cells with high efficiency. We have used this MC29 clone to screen a λphage/chicken DNA library for cellular sequences homologous to the viral MC29 sequence. The endogenous MC29 clone contains all MC29 specific sequences and a 1.3 kb cellular intervening sequence.

Project Description

Objectives:

The scope of this investigation is to delineate the relationship between virus gene expression and conversion of cells from normal to malignant state and to study the molecular anatomy of known tumor viruses and describe the mechanism by which subviral structures act in concert with cellular factors to regulate oncogenesis. The technique of molecular cloning, DNA sequence analysis, and site-specific mutagenesis will be used to implicate specific nucleotides in the transformation process.

Methods Employed:

A. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging. Isolation of phage of libraries containing virus-related sequences by hybridization of cDNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure. Isolation of cloned integrated proviral DNA sequences. Sucrose gradient-purified restriction fragments from a complete EcoRI digestion of Q5 DNA, ranging in size from 5 to 15 kb, were ligated to λgtWES•λB arms. After banding in CsCl, phage particles produced from the ligation reaction by in vitro packaging were adsorbed to Escherichia coli LE392, as described by Blattner et al., and spread on 150-cm agar plates at a density of 5,000 plaques per plate. Nitrocellulose filters were lifted from the plates and positive plaques were identified by hybridization to an AMV(MAV) [^{32}P]cDNA probe.

B. Construction of Recombinant Plasmids. Double-stranded cDNA of AMV genome was prepared. It was converted to perfect duplex molecules with blunt ends by digestion with single-strand nuclease S_1. It was then incubated with E. coli. DNA polymerase I. The product of the S_1-DNA polymerase reaction was ligated to Bam HI linkers with T4 DNA ligase. pBR322 was digested with Bam HI, treated with alkaline phosphotase. It was then ligated to double-stranded cDNA linked to Bam HI linkers.

C. Transformation and Identification of Recombinant Clones. Construction of chimeric plasmids and the transformation of E. coli X1776 by these plasmids was performed in a p2 physical containment laboratory. X1776 was transformed by a transfection procedure. Transformed colonies containing AMV sequences were identified by colony hybridzation. The colonies were screened with ^{32}P-labeled AMV cDNA. Strongly hybridized colonies were selected and replated; single colonies were picked and grown.

D. Sequences of Integrated Viral Transforming Genes and Large Terminal Redundancies (LTR). The "leuk" gene and the LTR from AMV clones were sequenced by using the chemical cleavage method of Maxam and Gilbert. DNA fragments were labeled on the 5'-end with γ^{32}P-ATP and polynucleotide kinase under standard conditions. The DNA sequences of the LTR from the MC29 clone was done by using the cloning and sequencing procedure with M13mp7.

E. Computer Analysis. Computer analysis of nucleotide sequences was performed by utilizing the computer program originally developed by Korn *et al.*

Major Findings:

A. Avian myelocytomatosis virus (MC29), a defective acute leukemia virus, has a broad oncogenic spectrum *in vivo* and transforms fibroblasts and hematopoietic target cells *in vitro*. We have used recombinant DNA technology to isolate and characterize the sequences which are essential in the transformation process. Integrated MC29 proviral DNA was isolated from a library of recombinant phage containing DNA from the MC29-transformed nonproducer quail cell line Q5. The cloned DNA was analyzed by Southern blotting of restriction endonuclease digests and by electron microscopic visualization of R-loops formed between the cloned DNA and MC29 or helper virus RNA. It was found that the 9.2 kb cloned DNA insert contains approximately 4 kb of viral sequences and 5.2 kb of quail cellular sequences. The viral sequences contain all of the MC20-specific sequences and 5' helper-related sequences, as well as part of the envelope region. The size of the cloned *Eco*RI fragment is the same as that of the major band in *Eco*RI-cleaved Q5 DNA that hybridizes to viral sequences. Transfection of the cloned DNA into NIH 3T3 cells revealed that the MC29-specific sequences are functional in that they induce foci of transformed cells with high efficiency.

B. We have used a proviral DNA clone of the avian acute leukemia virus MC29 to screen a λphage/chicken DNA library for cellular sequences homologous to the viral *mcv* sequence. A partial restriction map of three overlapping cellular DNA inserts, which span a region of over 30 kb in length, has been determined. Hybridization to probes derived from either the 5' half or 3' half of the viral *mcv* sequence is limited to a region of 7 kb or less (see below).

In order to determine the extent of sequence homology between the viral *mcv* sequence and the cellular *mcv* sequence, we have performed protection hybridizations using DNA from these c-*mcv* clones and ^{32}P-MC29 RNA. The hybrid which remains after treatment with RNase T_1 alone has been analyzed by RNA fingerprinting techniques. Most, if not all, T_1-oligonucleotides previously identified as being *mcv* specific are protected. Further analysis using both RNase A and T_1 has been used to determine any differences between the two related sequences.

C. Two virus-specific RNA species of 7.5 and 7.0 kilobases have been identified in avian myeloblastosis virus (AMV) by denaturing gel electrophoresis and blot hybridization analysis, and they were found to be in a 10:1 ratio. The individual RNAs directed the cell-free synthesis of the 76,000 dalton "gag" protein and the 180,000-dalton "gag-pol" protein, thereby demonstrating 5' sequence homology of approximately 4.9 kilobases between the two species. Synthesis of these two precursor proteins by the AMV genome indicates structural differences between AMV and other avian acute leukemia viruses. The two viral RNAs were transcribed into complete cDNA copies with AMV DNA polymerase. Linear proviruses were found to be 90-100% resistant to S1 nuclease. Analysis of single-stranded transcripts demonstrated two distinct species of 2.6 and 2.3 x 10^6 daltons, and analysis of duplexes formed from the single-stranded transcripts demonstrated species of 5.2 and 4.0 x 10^6 daltons.

D. Sheared chromatin prepared from chicken embryo fibroblasts and fibroblasts transformed by exogenous Rous sarcoma virus (Schmidt-Ruppin strain D) was separated by rate sedimentation on glycerol gradients into two components: fast-migrating (heavy chromatin fraction) and slow-migrating (light chromatin fraction). DNAs extracted from these fractions were assayed for proviral sequences by molecular hybridization using DNA complementary to the viral sequences. In uninfected cells, the endogenous complementary sequences were found to be equally distributed between heavy and light fractions. However, the newly integrated exogenous proviral sequences were found mostly in the light chromatin fraction in the transformed cells. Additionally, the light fraction is more sensitive to DNase I digestion and contains more material melting at low temperatures when compared with the heavy fraction. The results show that (i) distribution of endogenous proviral sequences is independent of chromatin conformation, and (ii) most of the newly acquired exogenous sequences are integrated within the host's chromatin fraction that exhibits properties of euchromatin. Because chromatin fragmentation and fractionation is accomplished without digestion with degrading enzymes, the chromatin fractions enriched in exogenous sequences remain intact and thus are suitable for further studies.

E. We have sequenced the LTRs of AMV and MC29. Both contain the same number of bases, have an identical U_5 region but completely different U_3. Both contain conserved signals such as inverted repeats, promoter site, polyadenylation site and small direct repeats.

F. We are completing the sequence of the AMV "leuk" sequences. An open frame has been detected coding for a 30,000 kb dalton protein.

Significance to Biomedical Research and the Program of the Institute:

It is clear that the formation, the maintenance and the expression of provirus are the central features of the life cycle of RNA tumor viruses. Many questions related to these aspects are unsettled. To elucidate the process of oncogenesis induced by these viruses, it is important to understand the structural organization of the transforming genes within the host chromosome and the process by which these genes are expressed and regulated.

Proposed Course:

Current research is conducted toward defining the mechanism of action of malignant transformation in cells. Efforts are concentrated towards:

1. Define specific sequences required for transformation by MC29. We already have shown that a fragment of 9.1 kb transforms 3T3 mouse cells. This assay will be used to clearly establish the minimum number of nucleotides required for transformation. The importance of "gag" LTR in the transformation process will clearly be established by deleting such regions and, in turn, testing its biological activity.

2. We have already initiated studies to synthesize the transforming protein, MC29, utilizing bacterial promoters in well-defined plasmids obtained from M. Ptashe. This will enable us to see if MC29-specific protein is formed where the MC29 sequences are expressed.

3. We are also involved in synthesizing the AMV transforming protein. The task here is relatively easy since we already know the nucleotide sequence.

4. We are presently characterizing endogenous chicken MC29 clones and six human clones, all isolated from human libraries.

Publications:

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Papas, T. S., Schulz, R. A., and Chirikjian, J. G.: Enzymatic synthesis of duplex DNA by avian myeloblastosis viral reverse transcriptase: In Papas, T. S.and Chirikjian, J. G. (Eds.): *Gene Amplification and Analysis,* Vol. II, *Analysis of Nucleic Acid Structure by Enzymatic Methods*. New York, Elsevier North-Holland, May, 1981 (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04963-06 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Studies on the Genome and Gene Products of Harvey and Kirsten Murine Sarcoma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	T. Y. Shih	Research Chemist	LTVG	NCI
OTHERS:	E. M. Scolnick	Chief	LTVG	NCI
	R. Ellis	Staff Fellow	LTVG	NCI
	Z. Selinger	Visiting Scientist	LTVG	NCI
	M. Furth	Staff Fellow	LTVG	NCI

COOPERATING UNITS (if any)
P. Gruss and G. Khoury, LMV, NCI
S. Orozlan, Biological Carcinogenesis Program, FCRC, Frederick, Maryland 21701
E. Chang and D. Lowy, DBCD, NCI

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Malignant transformation by the rat-derived Harvey and Kirsten sarcoma viruses was mediated by a p21 protein coded for by the virus genome. This p21 protein possesses biochemical activities of GTP-specific autophosphorylation and guanine nucleotide binding. Efforts were undertaken to characterize in more detail these activities, and their possible role in regulating the pKm protein kinase which phosphorylates the Na^+/K^+ATPase. Biosynthesis of p21 was studied by taking advantage of the elevated synthesis in the SV40-HaSV recombinants.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The scope of this project is to study the molecular virology of rat-derived RNA sarcoma viruses especially Harvey (Ha-MuSV) and Kirsten (Ki-MuSV) strains of murine sarcoma viruses. We have identified a gene product, p21, to be the transforming src protein coded for by these viruses. In this ongoing year, we have focused our primary efforts to further characterize the biochemical properties of this protein with the objectives of trying to understand the basic molecular mechanism of cell transformation directly caused by this viral gene product. From this baseline information, we hope to learn more about properties and function of the cellular homologue of this p21 src protein in normal cellular development and natural oncogenesis. The cellular homologue of p21 is widely present in all vertebrate species, including human, and is highly conserved during the evolution of vertebrate species.

Methods Employed:

(1) Immunoprecipitation: The p21 protein products were identified by immunoprecipitation of cell extracts labeled with ^{35}S-methionine using sera containing antibodies to the p21. The precipitated proteins were analyzed by SDS-gel electrophoresis followed by autoradiography. The p21 src protein could also be identified by a GDP-binding assay in which the cell extracts were incubated with [^{3}H] GDP and the nucleotide bound to p21 were quantitated following immunoprecipitation with antisera.

(2) Peptide analysis: Proteins labeled with either ^{35}S-methionine or ^{32}Pi were analyzed for their relationship by tryptic peptide mapping. Proteins precipitated by antisera were isolated by SDS-gel electrophoresis. Specific protein bands were eluted and digested with trypsin. The tryptic peptides were analyzed by 2° thin-layer electrophoresis and chromatography.

(3) Phosphoamino acid analysis: To analyze the amino acid residue phosphorylated by *in vivo* or *in vitro* phosphorylation reaction, the proteins were isolated by immunoprecipitation and SDS-gel electrophoresis. After acid hydrolysis, the phosphoamino acids were then analyzed on TCL plates using p-tyrosine, p-threonine and p-serine as markers.

(4) Phosphorylation and protein kinase activities: Purified p21 were assayed for its phosphorylation activities by incubation with ATP or GTP labeled with ^{32}P at its γ-phosphate. The phosphorylated proteins were detected by autoradiography following SDS-gel electrophoresis.

(5) Photoaffinity labeling: 8-azido-GTP labelled with ^{32}P at its γ-phosphate was covalently linked by UV irradiation to proteins with which the photoreactive analogue of GTP has affinity. The labeled proteins were then analyzed by SDS-gel electrophoresis.

Major Findings:

(1) GTP-specific autophosphorylation activity of Ha-MuSV p21. The purified p21^{src} protein of Ha-MuSV shows a quanine nucleotide-binding activity and, in addition, at elevated temperature an autophosphorylation activity at a threonine residue using as phosphoryl donor GTP or dGTP but not ATP or dATP. These biochemical activities are unique among those associated with transforming proteins of RNA-containing or DNA-containing tumor viruses. In contrast to the p21 purified by hydroxyapatite chromatography and phenylsepharose column, the p21 immunoprecipitated from crude cellular lysates with antisera can be autophosphorylated by ATP far better than GTP. Experiments indicated, however, that this phosphorylation by ATP might be an indirect one, i.e., through the nucleotide kinase which transferred γ-^{32}P from ATP to GDP bound to p21. Inclusion of an ATP/GTP regeneration system in the phosphorylation reaction greatly eliminated phosphorylation by ATP but not GTP. Therefore, the p21 autophosphorylation is very GTP-specific. Furthermore, phosphorylation sites *in vitro* and *in vivo* appeared to be the same. Tryptic peptide analysis of the phosphopeptides indicated a single phosphorylation site at a threonine residue. In addition to GTP, autophosphorylation can also be affected by GTP- γ -S, resulting in a thiophospho derivative of p21.

(2) Comparison of various forms of p21. Ki-MuSV and Ha-MuSV are two very similar rat-derived RNA sarcoma viruses both encoding a p21 protein of very similar size. Recent studies on the *src* genes of these two viruses, however, shows they both derived from a different family of proto-*src* sequences in the rat genome. To further compare their relationship, peptide analyses of these two proteins were performed. Out of a total of 29 tryptic peptides, 14 are identical, while 6 are specific to Ha-MuSV and 9 to Ki-MuSV. However, under a stringent hybridization condition, the nucleic sequences of these two *src* genes demonstrated little homology. Thus, conservation of primary amino acid sequence, perhaps due to conservation of its function, occurred during the evolution of these genes. Both Ki-MuSV p21 and Ha-MuSV p21 are all phosphorylated at a threonine residue, different from many forms of endogenous p21, such as the p21 highly elevated in a hemopoietic cell line (416B) and the p21 coded for by the proto-*src*$_{II}$ gene which contains three introns and different from the proto-*src*$_{I}$ gene which is colinear with the Ha-MuSV *src* gene. It will be very interesting in future studies to distinguish the function and biochemical activities of these endogenous cellular p21s from the viral p21.

(3) p21 synthesis in the SV40-Ha-MuSV recombinant. Recombinants of the Ha-MuSV *src* gene using the SV40 vector have been constructed. These recombinants express high levels of p21 in the infected AGMK monkey cells. The synthesis and biochemical activities of p21 synthesized by SV40-Ha recombinants are indistinguishable from p21 found in Ha-MuSV transformed cells. These recombinants will provide an invaluable source for the purification of this protein, study of its synthesis, sequence analysis of the p21 protein, and regulation of p21 gene expression.

(4) Photoaffinity labeling of p21. The purified p21 was photolabeled with ^{32}P-8-azido GTP. Although direct labeling of p21 was not observed, a strong labeling of a 40K protein was consistently observed. The binding to this 40K

protein was completely competed by ATP. This observation raised the possibility that this 40K protein might be the catalytic subunit of the recently identified pKm which phosphorylate the Na^+/K^+ ATPase.

Significance to Biomedical Research and the Program of the Institute:

Recent studies in the rat-derived Harvey and Kirsten sarcoma viruses, the widely studied Rous sarcoma virus and many other RNA viruses, such as Abelson murine leukemia virus and feline sarcoma virus, have firmly established that the transformation by these viruses was directly mediated by the transforming protein coded for by these viruses. These transforming proteins possess protein kinase activity of phosphorylating proteins at its tyrosine residue. Spector and Racker have recently identified a cascade of protein kinases regulating the activity of the Na^+/K^+ ATPase. Significantly, most of these transforming proteins in one way or the other regulate this kinase cascade through phosphorylation. Spector and Racker, in collaboration with Scolnick, have identified a 20K subunit of the pKm kinase to be homologous to the Ha-MuSV p21. This observation raised the possibility that the p21 may function as a regulatory subunit of pKm. GTP-specific autophosphorylation of p21 or its GTP/GDP binding activity may thus act as a switch to activate or inactivate the pKm activity. Perturbation of this cascade kinase system may be the fundamental mechanism of cell transformation by these viruses.

It has also been widely established that transforming genes of these viruses are derived from their cellular homologs which can be detected in most vertebrate species including human. It will be of ultimate importance to see what are the normal cellular functions these genes perform and their expression in natural oncogenesis. The understanding of the exact biochemical mechanisms of these viral proteins will be important to distinguish these viral transforming proteins from their normal cellular homologs, i.e., whether transformation is a simple overproduction of these proteins in virally infected cells, or whether there is subtle mutational alteration in its activities. Furthermore, the understanding of this biochemical mechanism will enable us to more rationally devise ways to intervene the transformation process and perhaps cancer chemotherapy. The identification of the precursor-product relationship of the Ha-MSV p21 may resemble the zymogenenzyme relationship and offers a possible point to intervene the p21 transforming activity.

Proposed Course:

The model that p21 may function as a regulatory subunit of pKm, the protein kinase which phosphorylates the Na^+/K^+ ATPase, will be explored. The various forms of the cellular homologs of p21 will be studied by biochemical techniques such as isoelectrofocusing. Attempts will be made to further purify the p21 and to study its role in pKm by reconstitution experiments between the catalytic subunits and various forms of possible regulatory subunits. Biosynthesis of p21 will be studied to determine possible intervention at this step. Use of SV40-Ha recombinants in this aspect of study will be explored. The role of p21 phosphorylation and GTP/GDP-binding activity in regulating p21 activities will be studied.

The phosphorylation site and nucleotide binding site will be determined. The amino acid sequence of p21 will be studied to compare with sequence deduced from DNA sequencing.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04970-05 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemistry of Cellular Transformation by Avian Sarcoma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. P. Bader Head, Cellular Transformation Section LTVG NCI

COOPERATING UNITS (if any)

R. Balaban Staff Fellow, Lab. of Kidney and Electrolyte Metabolism NHLBI

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Cellular Transformation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Malignant transformation by the avian Rous sarcoma virus is induced by a virus-coded protein which plays no role in virus reproduction. Attempts have been made to identify the metabolic function of this protein in transformation. Characteristic morphological and metabolic features of transformed cells have been used as a basis for examining possible functions of the transforming protein and a virus mutant which induces temperature-dependent transformation has been useful in distinguishing between direct and indirect effects of the protein. Using these methods, we have been able to eliminate two general metabolic areas, monovalent cation transfers and glycosylation reactions, from consideration as directly involved in transformation. On the other hand, differences in cytoskeletal proteins have been observed, and the possibility that they may directly bear on the transformation process is being pursued.

Project Description

Objectives:

To determine the biochemical function of the virus-coded protein responsible for the malignant transformation of cells by Rous sarcoma virus; to determine the primary physiological effects resulting from the functioning of this protein; to describe the sequence of metabolic changes which result in the altered metabolic profile characteristic of malignant cells; to distinguish metabolic changes necessary to the maintenance of the malignant state from those which are irrelevant.

Methods Employed:

(A) Transformation of cells in culture by avian and murine sarcoma viruses, morphological resolution of transformed cells by microscopy, resolution of cellular organelles by supravital staining.

(B) Isolation of cellular organelles, including microsomes, cell surface membranes, and nuclei.

(C) Quantitative chemical determinations of protein, and a variety of enzymes.

(D) Complement fixation, immunoprecipitation, immuno-affinity chromatography.

(E) Dextran and sucrose density gradients, polyacrylamide gel electrophoresis, starch gel electrophoresis, autoradiography, fluorography.

(F) Uptake of radioactive molecules into cells, incorporation of radioactive precursors into macromolecules, binding of radioactive molecules to specific cell-surface sites.

Major Findings:

Attempts have been made to determine the function of the virus-coded protein responsible for the malignant transformation induced by Rous sarcoma virus (RSV). The Bryan "high titer" strain of RSV (RSV-BH) induces a unique and characteristic morphological change in infected cells. A mutant of this virus (RSV-BH-Ta), isolated in this laboratory, induces temperature-dependent transformation. At 41° infected cells appear nontransformed, but after shifting to 37°, a variety of morphological and biochemical changes occur, resulting in a transformed phenotype indistinguishable from cells transformed by wild-type RSV-BH. We have identified several matabolic changes induced during transformation which require new RNA synthesis and protein synthesis, including increased glucose uptake and glycolysis, increased hyaluronic acid synthesis, decreased alkaline phosphatase activity, and decreased adenosine deaminase activity. These metabolic changes are preceded by morphological changes characteristic of RSV-BH transformation which occur even while RNA synthesis or protein synthesis is inhibited.

An increase in the rate of sodium ion uptake in RSV-BH transformed (CE-BH) cells, observed in preliminary experiments, prompted us to examine other features of sodium and potassium ions in transformed and nontransformed cells. Leakage of $^{22}Na^+$ into CE-BH cells was about double the rate of leakage into nontransformed chick-embryo cells, and the greater permeability of CE-BH cells was apparent in higher intracellular Na^+ concentrations. Experiments with cells exhibiting temperature-dependent transformation showed that both new RNA synthesis and protein synthesis were required for the acquisition of increased Na^+ permeability, suggesting that the change is an indirect effect of the virus-coded transformation-inducing protein. No differences in Na^+ uptake or intracellular Na^+ concentrations were found when cells transformed by another strain of RSV, the Schmidt-Ruppin strain, were compared to nontransformed cells.

Activation of (Na^+-K^+)-ATPase by artificially increasing the permeability of nontransformed cells to Na^+ was shown by an increased rate of uptake of $^{86}Rb^+$, a substitute for K^+. However, rates of $^{86}Rb^+$ uptake were indistinguishable in CE, CE-BH, and CE-SR cells. Also, equilibrium intracellular levels of $^{86}Rb^+$ were similar in transformed and nontransformed cells, as were observed in intracellular concentrations of K^+. No differences in (Na^+-K^+)-ATPase acitvity, as indicated by ouabain binding or temperature sensitivity, were observed. These studies argue against the possibility that nonvalent cations, or the activity of (Na^+-K^+)-ATPase, play a direct role in RSV-induced transformation, although the higher levels of Na^+ in CE-BH cells may be responsible for other distinguishing features of these cells.

A decreased efficiency of (Na^+-K^+)-ATPase activity has been implicated as the driving force for metabolic changes in malignant cells. Although experiments noted above suggested no obvious differences in (Na^+-K^+)-ATPase activity between transformed and nontransformed cells, the efficiency (amount of ATP required to pump 1 molecule of K^+) had not been examined. The relationship of ATPase activity to glycolysis and respiration can be utilized to examine ATPase efficiency. By devising a system which simultaneously measures K^+ uptake, O_2 consumption, and lactate production, we have been able to show a minor regulatory role of (Na^+-K^+)-ATPase on these functions, and preliminary estimates suggest that changes in (Na^+-K^+)-ATPase must be drastically greater that those observed in order to change cellular metabolism even in a subtle way.

Transformed cells have a higher glucose-uptake capacity than nontransformed cells. In nontransformed cells, glucose deprivation, or treatment with substances which activate (Na^+-K^+)-ATPase or mitochondrial ATPases, can induce an increased capacity for glucose uptake. Nonetheless, the glucose uptake capacity of CE-BH cells usually exceeds that which can be induced in nontransformed cells. Also, new uptake sites in transformed cells are generated through transcription and translation, whereas regulation of existing molecules is responsible for the occurrence of new sites in glucose-deprived or ATPase-activated cells. Lowered ATP (or high ADP) may be responsible for the glucose-deprivation phenomenon, and CE-BH cells contain lower amounts of ATP than CE cells. Also, CE-BH cells respond neither to glucose-deprivation nor ATPase activation by increasing glucose uptake capacity, and addition of large amounts of glucose, which suppresses glucose uptake capacity in nontransformed cells, has no such effect in CE-BH cells.

The relevance of the cytoskeleton to virus-induced transformation is under examination. Three differences in cytoskeletal proteins have been observed between CE cells and CE-BH cells: (1) Tubulin concentrations of transformed and nontransformed cells appeared similar, and no differences were observed in the proportions of tubulin in dimer and microtubule forms; however, two-to-five-fold differences in stability of dimers to cold treatment were observed. (2) Total amounts of actin were similar in transformed and nontransformed cells, but a form with affinity for microtubules is found in much lower levels in CE-BH cells than in CE cells. (3) A specific glycoprotein (m.w. 50,000) occurs in decreased amounts in CE-BH cells, and has properties similar to an actin molecule of lower molecular weight. These differences are being examined for their relationship to a recently observed difference in ability of cell extracts to form gels upon extended incubation.

The importance of glycosylation in virus-induced transformation also was studied. Transformation by both RSV-BH and RSV-SR was shown to develop under conditions which inhibit glycosylation of proteins. Treatment of cells with deoxyglucose (2 mM) or tunicamycin (0.1 μg/ml) decreased the incorporation of radioactive mannose or glucosamine into macromolecules, with little effect on the incorporation of leucine or uridine. After infection of cells by RSV-BH or RSV-SR, these treatments inhibited the production of infectious virus, although noninfectious particles containing viral RNA and structural proteins were produced. In the same cultures, morphological transformation developed unimpeded, and the increased capacity for glucose uptake developed. These results suggest that glycosylation is not an important feature of transformation induced by Rous sarcoma virus.

The glycosylation inhibitors also have been used to overcome resistance to infection induced by earlier infection of cells with a nontransforming retrovirus. When cells were infected with RAV-1 then treated with deoxyglucose, a secondary infection 24 hours later with transforming RSV-BH (RAV-1) or RSV-SR (A) was successful, while untreated cells were refractory to infection. Cells infected with RAV-1, then grown for several generations, also could be infected with RSV-BH (RAV-1) if pretreated with deoxyglucose. These experiments suggest that such treatments could generally be useful in overcoming glycoprotein-induced interference with virus infection.

Significance to Biomedical Research and the Program of the Institute:

The resolution of the function of a protein responsible for malignant transformation would be a major advance in our understanding of the nature of cancer cells. Recognition of a primary physiological imposition on the cell which converts that cell to a malignant form, and the identification of characteristic metabolic changes induced during the development of transformation, would allow a description of the entire sequence of metabolic events which culminate in malignancy. Such studies could lead to a determination of metabolic events necessary to the maintenance of the malignant state, but not to normal metabolism, and the possibility of therapeutic intervention in such metabolism.

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Proposed Course:

To determine the primary function of the RSV-BH protein responsible for malignancy, and to describe the sequence of metabolic changes culminating in the phenotype characteristic of RSV-BH transformed cells. Studies on cytoskeletal elements will be pursued, and attempts will continue to identify the primary substrate of the virus-coded protein.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT**	**PROJECT NUMBER** Z01 CP 04980-04 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Proteins Associated with the Biological Effects of Type C Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.K. Ruscetti	Senior Staff Fellow	LTVG	NCI
OTHERS:	E.M. Scolnick	Chief	LTVG	NCI
	D.L. Linemeyer	Senior Staff Fellow	LTVG	NCI
	A.I. Oliff	Research Associate	LTVG	NCI
	L. Wolff	Postdoctoral Fellow	LTVG	NCI
	R.H. Bassin	Section Head	LTVG	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 3.0	**PROFESSIONAL:** 1.0	**OTHER:** 2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Both the spleen focus-forming virus (SFFV) and the Friend murine leukemia virus (F-MuLV) induce erythroleukemia in susceptible strains of mice. Two strains of SFFV, having different biological effects, exist: a polycythemia-inducing strain (FVP) and a mild anemia-inducing strain (FVA). Both FVP and FVA strains of SFFV encode similar envelope-related proteins, $gp52^{env}$, but differ in the post-translational processing of these proteins. The $gp52^{env}$ of FVP-SFFV is processed to a 65,000 dalton, galactose-containing protein that eventually appears on the cell surface. In contrast, the majority of the $gp52^{env}$ encoded by FVA-SFFV is not further processed to a higher molecular weight, galactose-containing form and cannot be detected on the cell surface. The Friend MuLV induces in newborn mice an erythroproliferative disease associated with severe anemia. Studies have indicated that Friend MCF virus is a crucial intermediate in the induction of this disease and that certain mouse strains endogenously express a novel envelope glycoprotein, related to MCF gp70, which confers resistance to this disease. Studies are presently being carried out to determine how spleen focus-forming and Friend MCF viruses interfere with erythropoiesis and why certain strains of mice are resistant to disease induced by these viruses.

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Project Description

Objectives:

(1) To correlate differences in biological effects of different strains of SFFV with biochemical properties of the proteins encoded by them and to determine the role that these proteins play in altering the erythroid differentiation pathway.

(2) To document an association between leukemia induced by certain ecotropic viruses and the formation of recombinant MCF viruses and to understand the mechanism by which certain strains of mice, or mice of a certain age, are rendered resistant to disease induced by these viruses.

Methods Employed:

(1) Preparation of specific antisera to viral-encoded proteins, including the preparation of monoclonal antibodies.

(2) Use of specific antisera to precipitate proteins from cytoplasmic extracts of metabolically labeled virus-infected cells and tissues.

(3) Analysis of immune precipitates by SDS-polyacrylamide gel electrophoresis and autoradiography.

(4) Use of specific antisera in competition radioimmunoassays to detect viral-encoded proteins in various virus-infected cells and tissues.

(5) Analysis of viral protein expression in individual cells by immunofluorescence.

Major Findings:

(A) Studies on SFFV:

(1) It was previously shown that polycythemia-inducing (FVP) and anemia-inducing (FVA) strains of the spleen focus-forming virus (SFFV) code for closely related proteins, a 52,000 dalton envelope-related glycoprotein and a 45,000 dalton gag-related protein. Tryptic peptide analyses were carried out on the the envelope-related proteins encoded by these viruses and the results showed that differences exist in the envelope-related proteins encoded by various polycythemia-inducing strains of SFFV as well as between polycythemia and anemia-inducing strains. So far, these differences cannot be correlated with the differences in the biological effects of these viruses. However, when one compares the post-translational processing of the envelope glycoproteins encoded by these viruses, both a quantitative and qualitative difference between the proteins encoded by FVA and FVP strains can be found. FVP strains of SFFV encode a gp52 which is processed to a galactose-containing 65,000 dalton molecular weight glycoprotein which eventually appears on the cell surface. In contrast,

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the majority of the gp52 encoded by FVA-SFFV is not further processed to a higher molecular weight, galactose-containing form and cannot be detected on the cell surface.

(2) In order to obtain immunological reagents reactive with defined determinants on SFFV gp52, hybridoma cell lines secreting monoclonal antibodies to gp52 were prepared by fusing SP-2 mouse myeloma cells with lymphocytes from rats immunized with SFFV/NRK nonproducer cells or semipurified gp52. One of these monoclonals has been extensively characterized using immune precipitation and polyacrylamide gel electrophoresis. This antibody is specific for a determinant on both FVA and FVP-SFFV-encoded gp52 and is cross-reactive with the gp70's of a number of MCF viruses and a variety of xenotropic and amphotrophic virus isolates. However, it fails to react with the gp70's of any ecotropic virus tested, with the exception of a single stock of Moloney MuLV. It also fails to react with the gp70 encoded by the feline leukemia virus.

(B) Studies on F-MuLV

(1) Data accumulated in the past year are consistent with the hypotheses that MCF viruses play an important role in the generation of an erythroproliferative disease developing after injection of certains strains of newborn mice with ecotropic Friend MuLV and that resistance to this disease is mediated through the endogenous expression of an MCF/xeno-gp70-related protein that interferes with the replication and spread of MCF viruses. These data are supported by the following observations:

(i) After infection with F-MuLV, only 6/13 strains of mice developed disease, and studies with crosses between susceptible and resistant strains indicated that resistance was dominant. While F-MuLV was shown to replicate equally well in all strains tested, viruses coding for MCF-specific viral envelope proteins could be detected only in the spleens of mice from strains that were susceptible to F-MuLV-induced disease and not in the spleens of mice from strains that are resistant to this disease.

(ii) A Friend MCF virus isolated from the spleen of an F-MuLV-infected mouse from a susceptible strain induced the same erythroproliferative disease when injected as an appropriate pseudotype into mice from susceptible, but not resistant, strains of mice.

(iii) Resistant, but not susceptible, strains of mice endogenously express MCF/xeno-related envelope glycoproteins which may be responsible for resistance by blocking receptors for MCF viruses.

These results not only indicate that Friend MCF virus is a crucial intermediate in the induction of disease by F-MuLV, but suggest that a novel gene, either an MCF/xeno-related envelope gene or a gene controlling its expression, is responsible for resistance to erythroleukemia induced by F-MuLV.

(2) In order to test the possibility that the novel MCF/xeno-related envelope glycoprotein endogenously expressed in certain strains of mice is responsible

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(4) Murine erythroleukemia cell lines have been previously isolated from the spleens of mice infected with the Friend virus complex, containing both F-MuLV and SFFV. Dr. Allen Oliff, in this lab, developed several erythroleukemia cell lines from the spleens of NFS mice inoculated as newborns with F-MuLV alone. The lines were examined by immunofluorescence for the presence of spectrin, a marker specific for cells belonging to the erythroid lineage, and were shown to be positive. The cell lines were also examined by pulse-labeling and immune precipitation with specific antisera for viral protein expression and were shown to be expressing both F-MuLV and Friend MCF gp70 but no SFFV-encoded proteins. These results indicate that erythroleukemia cell lines can be established in the absence of SFFV.

(5) Studies were carried out in collaboration with Dr. Paul Hoffman, Dept. of Neurology, Medical U. of S. Carolina, Charleston, S.C., to investigate the role of MCF viruses in both lymphomas and neurologic disease induced by a wild mouse ecotropic virus (Cas-Br-M). We showed that MCF gp70 could be detected in the spleens of infected mice that would later develop lymphomas and suggested that such recombinant viruses play a role in the non-thymic lymphomas induced by this wild mouse ecotropic virus. No such association was shown between the expression of MCF viruses in the brain and neurologic disease, suggesting that MCF viruses may not play a role in the development of hind limb paralysis included by this ecotropic virus.

Significance to Biomedical Research and the Program of the Institute:

Understanding the proteins that are responsible for the biological effects of RNA tumor viruses is of great importance. The erythroleukemia-like diseases induced in mice by the spleen focus-forming virus and the Friend leukemia virus are associated with the expression of specific viral proteins which are products of the spleen focus-forming virus and a generated recombinant MCF virus, respectively. It is hoped that through further characterization of the proteins encoded by these murine leukemia viruses a correlation between biochemical parameters and biological effects will be found. Using reagents and information gained from studying these model systems may ultimately help us to develop immunoassays which would detect cross-reacting leukemia-specific proteins in primate species such as man. In addition, the study of the mechanisms of resistance to virus-induced leukemia in the mouse will also have relevance for the treatment of human leukemias.

Proposed Course:

(A) Studies on SFFV:

(1) Additional studies will be carried out to determine whether the difference in post-translational glycoprotein processing in cells infected with either FVA or FVP strains of SFFV is responsible for the different biological effects of these viruses. These will include analysis of the biological effects of FVP-SFFV under conditions where glycoprotein processing is inhibited as well as studies with putative mutants of molecularly cloned FVP-SFFV that biologically resemble FVA-SFFV.

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(2) The gp52 from rat cells nonproductively infected with the spleen focus-forming virus will be further purified using affinity and ion-exchange chromotography as well as by gel filtration. Monoclonal antibodies to gp52 will be utilized for immunoaffinity chromatography. The purified protein will be used in the development of specific immunoassays as well as in assays (such as microinjection) to determine its effect on hematopoietic cells.

(3) Immunofluorescence assays are being developed to study viral protein expression in individual cells. We hope to be able to determine if viral proteins are expressed in all hematopoietic cells or only in certain cells that represent the targets of the virus. Immunofluorescence assays will also be useful in detecting viral proteins when only a few cells in a culture are positive, which is not possible using extracts of an entire culture.

(B) Studies on F-MuLV:

(1) Studies on the resistance of various strains of mice to F-MuLV-induced disease will be continued in an attempt to determine the basis for this resistance. This will include additional in vitro studies carried out in collaboration with Dr. R. Bassin of this laboratory to further document the proposed viral interference model for resistance, as well as genetic studies carried out in collaboration with investigators at NIAID to determine the number and location of genes involved in this resistance.

(2) Additional studies will be carried out in an attempt to determine why adult NIH Swiss mice are resistant to erythroleukemia induced by F-MuLV. Preliminary data indicate that an antibody response against the generated MCF viruses may be involved in this resistance and studies will be carried out to determine if macrophages or natural killer cells may also be involved.

(3) Studies will be carried out in collaboration with Dr. D. Hankins of this laboratory to determine the effects of Friend MCF viruses on erythropoiesis in vitro.

Publications:

Linemeyer, D.L., Ruscetti, S.K., Menke, J.G., and Scolnick, E.M.: Recovery of biologically active SFFV from molecularly cloned SFFV-pBR322 circular DNA by cotransfection with infectious type C retroviral DNA. J. Virol. 35: 710-721, 1980.

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Linemeyer, D.L., Ruscetti, S.K., Scolnick, E.M., Evans, L.H., and Duesberg, P.H.: Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA. Proc. Natl. Acad. Sci. USA, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04986-04 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mouse Mammary Tumor Virus-induced Mammary Carcinogenesis; Structure and Regulation of Mammalian Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Hager	Head, Viral Immunogenetics Section	LTVG	NCI
OTHER:	R. Wolford	Microbiologist	LTVG	NCI
	D. Berard	Microbiologist	LTVG	NCI
	L. Donehower	Chemist (graduate student)	LTVG	NCI
	A. Huang	Chemist (graduate student)	LTVG	NCI
	D. Wheeler	Chemist (graduate student)	LTVG	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Tumor Virus Genetics

SECTION
Viral Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	1.0	4.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new gene has been discovered as an open reading frame (ORF) encoded within the long terminal redundancy (LTR) of mouse mammary tumor virus (MMTV). The ORF is also maintained within the 3' LTR of an MMTV endogenous genome (unit II). Polypeptides specific to this sequence have been synthesized in bacteria using molecular clones of MMTV and bacterial expression plasmids. Previously undetected transcripts expressed from the MMTV genome have been identified in MMTV-transformed cells and MMTV-induced tumors; included is a 2.5 kb transcript that is regulated independently from the classical 35s and env messages. Molecular chimeras between MMTV regulatory sequences and heterologous genes (p21 transformation gene from Ha-MuSV) have been constructed. Two classes of transfectants produced with these constructions have been identified. In one class, the expression of the p21 transformation function is not hormonally regulated, and apparently derives from a non-MMTV cellular promotion sequence. In the second class, the level of p21 protein is under glucocorticoid regulation.

PHS-6040
(Rev. 2-81)

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Project Description

Objectives:

(1) Mechanisms by which transformation of mammary cells is initiated by mouse mammary tumor virus are to be elicited.

(2) DNA sequences (genes) specifically involved in mammary tumorigenesis are to be identified. Potential sequences would include gene(s) encoded by the MMTV genome, cellular onc sequences that become associated with and regulated by the MMTV genome in transformed cells, or cellular sequences activated indirectly in tumor cells.

(3) The regulation expression of retroviral genomes is to be explored with a view to understanding the mechanisms by which the transcription of these sequences are controlled. Both in vivo and in vitro systems will be utilized in studying this problem. Particular attention will be given to understanding the novel regulation of MMTV expression by glucocorticoid hormones.

(4) Genetic engineering of viral and cellular DNA is to be employed as a means of identifying sequences involved both in oncogenic transformation and in regulation of expression.

Methods Employed:

(1) Molecular recombinants are isolated by inserting restricted DNA fragments, either from cellular DNA or viral replicative intermediates, into plasmid or bacteriophage lambda vectors.

(2) Physical mapping of inserts is accomplished by restriction endonuclease digestion, followed by gel electrophoresis, Southern transfer, and in situ hybridization with specific cDNA's. Regions of particular interest are subjected to DNA sequence analysis by standard methods.

(3) Identification and characterization of RNA transcripts is performed by the standard techniques of Northern analysis using molecularly cloned DNA probes, and S1 nuclease mapping.

(3) Biological activity of recombinant or cellular DNA is determined by transfecting the DNA onto appropriate recipient cells and scoring for the development of the phenotype in question.

Major Findings:

(1) Structure of MMTV

The genetic coding capacity of the MMTV genome has been explored by DNA sequence analysis of recombinant clones containing various regions of the genome.

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A new gene has been discovered to be encoded, at least partially, within the long terminal redundancy (LTR) of infectious C3H-S virus. This is a novel feature of MMTV among retroviruses. No other LTR has been found to specify protein coding information. The presence of the LTR open reading frame (ORF) has also been identified within the 3' LTR of an MMTV genome endogenous to C3H mice (unit II), further substantiating the existence of a previously undetected MMTV gene. DNA fragments containing the LTR-ORF have been inserted into a bacterial plasmids containing a strong bacterial transcription and translation initiation region. Polypeptides have been detected in bacterial cells transformed with these plasmids that correspond to the LTR-ORF. Antibodies raised against the bacterial extracts have been shown to specifically precipitate the LTR-ORF protein. The expression of this novel MMTV gene in MMTV-infected mammalian cells is under study. The identification of the actual gene product will be the first step toward understanding the biological role of this novel gene.

(2) MMTV transcripts in viral-transformed cells

An extensive analysis of MMTV encoded transcripts has been undertaken to identify novel messages encoding potential transformation gene products. Several unusual messages have been identified, including a 2.5 kb RNA whose expression is regulated independently with respect to the 9 kb genomic and 3.8 kb envelope transcripts. The relationship of any of these transcripts to possible transformation functions is under investigation.

In an alternate approach, a library of molecular clones containing all cellular sequences downstream (3' distal) to newly integrated MMTV genomes in a tumor cell line has been constructed. A strategy for the rapid preparation of radio-labeled probes specific to the downstream cellular sequences has been developed. Preliminary results suggest that cellular genes that have been activated as a result of upstream integration (promoter insertion) can be detected by this approach. Again, the possible common expression of these RNA's in MMTV induced tumors and potential involvement as cell onc genes is under study.

(3) Regulation of retroviral expression

A major goal of this program over the past two years has been to prepare molecular chimeras between MMTV transcription regulatory regions and viral or cellular genes for which biological selection assays are available. This would circumvent the problems of low infectivity and lack of in vitro transformation that have plagued the study of MMTV transformation and regulation since its discovery. A primary candidate in our constructions has been the p21 gene of Ha-MuSV. Expression of this gene under the control of the hormone-regulated MMTV promoter would bring the enormously useful tool of genetic selection at the cellular level into play in this system. We have now succeeded in identifying two classes of cells transformed with this type of construction. In one class, the MMTV LTR has apparently served as an integrating element, and the hormone-nonresponsive expression of the p21 gene is under the control of another non-MMTV promoter. In the second class, the level of p21 expression is hormone regulated; the presumed initiation at the MMTV promoter is currently being established. We now have available a biological system in which we can introduce known deletions or mutations into the MMTV regulatory sequences, and identify those elements involved in the hormone responsive mechanism. Alternatively, mutations in the hormone response can be selected at the cellular level vis-a-vis the transformation phenotype, and the mutated elements identified at the molecular level.

Significance to Biomedical Research and the Program of the Institute:

The results produced thus far in this project indicate the validity of a new approach to the study of RNA tumor virus infection, replication, and transformation. The application of recombinant DNA technology to the study of mouse mammary tumor virus regulation and transformation has yielded insights that would have been impossible to obtain by previous approaches. The discovery of a new MMTV-encoded gene opens up the possibility of its potential involvement either in the transformation process, or the unique hormone-responsive regulation of MMTV. The similarity between MMTV-induced carcinomas and hormone-responsive human breast cancer suggests the obvious importance of understanding the fundamental mechanims involved in these biological events. The successful development of molecular chimeras in which the unique hormone regulatory events can be coupled to a selectable gene provides us with powerful genetic tools for probing, at the cellular and molecular level, the processes involved in MMTV regulation and transformation. The knowledge we gain from this approach will undoubtedly prove pivotal in understanding the expression and regulation of transformation sequences in human disease.

Proposed Course:

We will extend the structural and biological analysis of MMTV transformation and regulation with a view toward correlation of structural sequence with biological function. Expression of the newly discovered MMTV gene will be analyzed at the RNA and protein levels, and efforts will be made to probe its biological function. Attempts to identify transformation genes involved in mammary carcinogenesis, either associated with MMTV or indirectly active, will continue. The analysis of retroviral transcriptional regulation will proceed in *in vitro* reconstruction experiments and in the newly developed *in vivo* systems in which the expression of heterologous genes can be linked directly to molecularly cloned retroviral promoter regions.

Publications:

Donehower, L.A., Andre, J., Berard, D. S., Wolford R. G., Hager, G. L.: Construction and characterization of molecular clones containing integrated mouse mammary tumor virus sequences. *Cold Spring Harbor Symp. Quant. Biol.* 44: 1153-1159, 1980.

Chang, E.H., Maryak, J. M., Wei, C.-M., Shih, T. Y., Shober, R., Cheung, H. L., Ellis, R. W., Hager, G. L., Scolnick, E. M., and Lowy, D. R.: Functional organization of the Harvey murine sarcoma virus genome. *J. Virol.* 35: 76-92, 1980.

Donehower, L.A., Huang, A., and Hager, G. L.: Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy. J. Virol. 37: 226-238, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04987-04 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Study of the Molecular Basis of Disease Induced by the Spleen Focus-Forming Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.L. Linemeyer	Senior Staff Fellow	LTVG	NCI
OTHER:	E.M. Scolnick	Chief	LTVG	NCI
	S. [illegible] Ruscetti	Senior Staff Fellow	LTVG	NCI
	W.D. Hankins	Cancer Expert	LTVG	NCI

COOPERATING UNITS (if any)

L. Evans and P. Duesberg	Univ. of California, Berkeley, CA
M. Gonda	Frederick Cancer Research Center, Frederick, MD

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Friend strain of the spleen focus-forming virus (SFFV), a replication-defective murine retrovirus, induces a rapid proliferation of erythroid precursor cells when it is injected as a pseudotype into susceptible mice. Two proteins encoded by SFFV have been identified, a gag gene-related protein and an env gene-related protein, gp52. In order to demonstrate which area(s) of the genome is responsible for the induction of disease, a genetic analysis of this virus has been performed. The gp52 protein is expressed in fibroblast cells after transfection and rescue of biologically active, molecularly cloned SFFV DNA. Evidence using two molecularly cloned subgenomic fragments of SFFV DNA and cloned in vitro mutagenized SFFV DNA indicates that the sequences necessary for the erythroproliferative disease and those encoding the gp52 are present in the same part of the viral genome; thus demonstrating that the gp52 is required for induction of the disease.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The purpose of this project is to identify the gene(s) responsible for the induction and/or maintenance of the erythroproliferative disease induced by the Friend strain of the spleen focus-forming virus (SFFV), and to examine this gene(s) and its gene product(s) to determine the role it plays in pathogenesis.

Methods Employed:

(1) Subgenomic fragments of SFFV DNA have been molecularly cloned in *E. coli* using the plasmid vector pBR322.

(2) The cloned DNA has been transfected into fibroblast cells and rescued by co-transfection of molecularly cloned and infectious helper viral DNA.

(3) The biological activity of the DNA has been monitored by injecting adult NIH Swiss mice with the virus produced after transfection and rescue of the DNA.

(4) The cloned DNA-mediated protection of viral genomic RNA labeled with radioactive phosphorous has been examined by two dimensional oligonucleotide fingerprinting analyses.

(5) The expression of viral encoded proteins has been examined by immune precipitation using various specific antisera.

(6) *In vitro* mutagenesis of the cloned DNA has been performed by insertion of molecular restriction linker sequences into random sites of the DNA. These mutated DNAs have been cloned and analyzed for biological activity.

(7) Heteroduplex analysis has been performed between DNA of the Friend helper virus and DNA of SFFV.

(8) Small fragments of viral DNA have been molecularly cloned for use as virus-specific hybridization probes.

Major Findings:

(1) A 3.0 kbp subgenomic fragment of SFFV DNA containing *env* gene sequences and the long terminal repeat sequences (LTR) of the viral DNA has been molecularly cloned. Heteroduplex analysis of this fragment and the 3' half of the parental Friend helper virus DNA has shown that the fragment contains the non-Friend virus sequences of SFFV acquired by recombination with an endogenous xenotropic-like virus.

(2) The 3.0 kbp fragment has been shown to be biologically active by transfection of the DNA and rescue from fibroblasts leading to the production of a virus which can induce an erythroproliferative disease in mice. This SFFV biological activity can be rescued from the transfected cells by co-transfection with Friend helper virus DNA or with infectious DNA of other helper viruses, such as Moloney or wild mouse amphotropic virus, which do not themselves induce an erythroid disease.

(3) A single cell clone has been obtained after cotransfection of fibroblasts with the 3.0 kbp DNA fragment and Moloney helper virus DNA. This single cell clone has been used to demonstrate that the SFFV-encoded gp52 is expressed in the transfected fibroblasts, and that the virus produced after the co-transfection can cause the production of erythroid bursts _in vitro_ when infected onto bone marrow cells.

(4) A smaller, 2.4 kbp, subgenomic fragment of SFFV DNA has also been molecularly cloned. This fragment is derived from the _env_ gene region and contains the LTR; however, it lacks 0.6 kbp from the 5' side of the _env_ sequences contained in the 3.0 kbp DNA clone. SFFV genomic RNA oligonucleotide protection experiments demonstrate that this smaller fragment, like the 3.0 kbp fragment, contains the SFFV-specific sequences which are not found in the parental Friend helper virus. This smaller fragment also retains the biological activity of SFFV. Further results indicate that the gp52 is also encoded within this 2.4 kbp fragment.

(5) A large number of deletion mutations have been randomly produced in the full-length clone of SFFV DNA by _in vitro_ mutagenesis. Eighteen molecular clones of the mutated DNA have been analyzed by restriction enzyme digestion to map the site of mutation; and these clones have been assayed for biological activity. Deletions of sequences in the _gag_ gene region do not affect biological activity. SFFV disease can not be demonstrated using a mutant clone which has only _env_ sequences deleted. Deletions of the LTR retain the biological effect but generally result in an increased latency of the disease. These results show that the _env_ gene sequences are required for SFFV disease. In no case has SFFV disease been observed without the expression of the _env_ gene product gp52.

(6) A 0.6 kbp subgenomic fragment of SFFV _env_ sequences has been cloned and used as a probe which is specific for SFFV and does not detect the ecotropic helper viruses used for rescue in the cotransfections. As expected, probe made from this fragment also hybridizes to mink cell focus-inducing (MCF) and xenotropic viruses, and is being used to study the formation of MCF viruses.

Significance to Biomedical Research and the Program of the Institute:

An understanding of the genetics of leukemia viruses is crucial to understanding the biological activity of these viruses. Molecular clones of various viral DNA species offer the most practical means to study the structure and function of viral genes. This will allow us to determine which viral proteins are responsible for the induced pathogenicity. An analysis of their mechanism of action can then be initiated. Cloned DNA from various viruses can also be used in recombination experiments to study how viruses are formed and to study how different genes interact.

Lastly, molecularly cloned subgenomic fragments of DNA are being used to generate specific probes which, in turn, can be used to detect and isolate related retrovirus genes in other species including man.

Proposed Course:

(1) The nucleotide sequence of the 3.0 kbp fragment will be determined to examine the precise initiation and termination sites of the gene encoding gp52. The sequence data will also be used to determine whether any other open reading frames exist in this SFFV DNA fragment that could encode another gene product which has not yet been detected Nucleotide sequencing will also be performed on crucial mutants to determine the exact site of the deletion.

(2) Using the information from the subgenomic fragment and mutant analyses, a specific 1.4 kbp fragment will be molecularly cloned. This fragment should represent exclusively the entire gp52 coding sequences.

(3) Single point mutations will be produced in the sequences of SFFV DNA responsible for erythroid disease induction. These mutations will be analyzed to study their affect on the function of the pathogenic gene product. They will also be used in an attempt to generate temperature-sensitive mutants to examine the maintenance of the SFFV disease.

(4) Gene transfer in hematopoietic cells will be attempted to develop an *in vitro* assay for SFFV DNA and to investigate whether SFFV alone, without a helper virus, can cause the proliferation of erythroid precursor cells.

(5) Using the DNA probes specific to SFFV, assays which could detect homologous sequences in various mouse and non-mouse cells will be developed. These SFFV-related endogenous sequences will be analyzed and potentially cloned to determine the nature of the sequences with which Friend helper virus may have recombined to give the pathogenic SFFV.

(6) Using cloned fragments of DNA from different viruses we will attempt to construct new recombinant viruses *in vitro* and analyze them to study the mechanism of formation of a pathogenic virus.

Publications:

Linemeyer, D. L., Ruscetti, S. K., Menke, J. G. and Scolnick, E. M.: Recovery of biologically active spleen focus-forming virus (SFFV) from molecularly cloned SFFV-pBR322 circular DNA by co-transfection with infectious type C retroviral DNA. *J. Virol.* 35: 710-721, 1980.

Oliff, A., Linemeyer, D., Ruscetti, S., Lowe, R., Lowy, D. and Scolnick, E.: A subgenomic fragment of molecularly cloned Friend murine leukemia virus (F-MuLV) DNA contains the gene(s) responsible for F-MuLV induced disease. *J. Virol.* 35: 924-936, 1980.

Ruscetti, S., Linemeyer, D., Troxler, D. and Scolnick, E.: Characterization of proteins coded for by the spleen focus-forming virus. In Yohn, D. S., Lapin, B. A., and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research. Elsevier, North Holland, 1980, pp. 323-324.

Linemeyer, D. L., Ruscetti, S. K., Scolnick, E. M., Evans, L. H. and Duesberg, P. H.: Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA. Proc. Natl. Acad. Sci. USA 78: 1401-1405, 1981.

Chattopadhyay, S. K., Oliff, A. I., Linemeyer, D. L., Lander, M. R. and Lowy, D. R.: Genomes of murine leukemia viruses isolated from wild mice. J. Virol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05117-02 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Genetic Analysis of Retroviruses: Control of Replication Transformation and Leukemogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. N. Tsichlis Expert, Viral Biochemistry Section LTVG NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oncogenesis by nontransforming type C retroviruses is due to activation of endogenous transforming genes. The work presented in this progess report addresses the general questions of the mechanism by which activation of the endogenous genes occurs, and the specificity of activation of a particular gene, for a particular target.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The overall objectives of this project are to define the role of specific regions of the genome of nontransforming type C retroviruses in the activation of endogenous transforming genes during the process of virus induced oncogenesis, and to study the tissue specificity of the transforming gene activation.

The specific goals are:

(1) To define the role of the U_3 region of the viral genome in integration and transcription.

(2) To study the mechanism by which the viral U_3 region determines the oncogenicity of nontransforming type C retroviruses.

(3) To study the role of regions of the viral genome other than the U_3 region in oncogenesis.

(4) To identify the endogenous transforming genes activated in various types of virus-induced malignancies.

(5) To analyze the transforming gene of the avian myelocytomatosis virus (MC29), the endogenous counterpart of which has been shown to be activated in virus-induced B cell lymphoid malignancies in chickens.

Major Findings:

1. RAVO and NTRE7 (a recombinant that is genetically identical to the endogenous virus of chicken RAVO, except at the U_3 region which is derived from td P7RSV-B) were compared regarding their growth properties in tissue culture. The comparison suggested that the difference in growth between the two viruses is a phenomenon that can be divided in two stages. The early stage from day 2-5 is characterized by marked differences in growth with NTRE7 reaching levels 10^2 - 10^3 times higher than those of RAVO. The late stage of chronically infected cells is characterized by modest differences in growth with the NTRE 7 reaching levels 5-10 times higher than those of RAVO. The growth pattern of these two viruses suggests that the role of the U_3 region in viral growth is a process that might involve more than one phenomena. While for example the late stage might be due to differences in the efficiency of transcription initiated at the U_3z^{cx} vs that initiated at the U_3^{end} region, the early stage could be explained on the basis of differences in the efficiency of integration of the two genomes within the cellular DNA.

In order to study this problem we have now molecularly cloned both RAVO and NTRE7 into lamda 3A Δ lac and subcloned them into pBR322. The overall plan and the reasoning by which the RAVO and the NTRE7 clones would be very useful in understanding this problem, are described under the proposed course.

2. Based on genetic evidence, we originally proposed a model according to which retrovirus induced oncogenesis is due to integration of the virus next to a potentially transforming gene. This gene is then transcribed efficiently from the viral promoter that resides within the U_3 region. (Tsichlis, P. N. and Coffin, J. M., J. Virol. 33:238-249, 1980; Tsichlis, P. N., and Coffin, J. M., Cold Spring Harbor Symp. on Quanti. Biol. Vol. XIIV "Viral Oncogenes" p. 1123, 1979). Evidence originating from this as well as other laboratories indicates that this model is correct at least in the majority of cases. W. Hayward has shown this to be the case in ALV induced B cell lymphoid malignancies in chicken. Working within the avian system, we have shown that in carcinomas and fibrosarcomas induced in chickens by NTRE 7 there are RNA transcripts that fulfill the criteria for cellular transcripts induced by the viral promoter. These transcripts are probably involved in oncogenesis even though we have not as yet formally shown that the gene transcribed is a transforming gene. (P. Tsichlis, H. Robinson and J. Coffin, in preparation.)

In order to extend these observations in another system and study the universality of the model, we have examined the RNA transcripts present in rat thymomas induced by MoMuLV. In this system we have shown again cellular transcripts induced by the viral promoter. However, this class of transcripts was observed in only 50% of the tumors. (P. Tsichlis, L.F. Hu , and T. Wood, in preparation.) What happens in the other 50% of the tumors is a matter of speculation at this point.

3. NTRE7 is a recombinant identical to the endogenous virus of chicken RAVO except at the U_3 region which is derived from Pr RSV-B. Even though the exact crossover that generated NTRE7 has not as yet been defined by sequencing, we know by comparing oligonucleotide maps that NTRE7 differs from other exogenous viruses in a region that maps to the left of the U_3 region, which in the case of this recombinant is derived from the endogenous virus genome. In order to determine the role of this region in oncogenesis, RAVO and NTRE7 were injected into chickens and their oncogenic potential was compared to that of the subgroup E exogenous viruses. This experiment was done in collaboration with Dr. H. Robinson. While RAVO was not oncogenic, NTRE7 caused several types of malignancies as expected. However, even though NTRE7 and RAV60 recombinants appeared to be almost identical in their ability to induce a variety of tumors, the ability of NTRE7 to induce B cell lymphomas was markedly reduced. This finding strongly suggests that a region that maps upstream from U_3 plays a major role in determining the target specificity of the virus (H. Robinson, P. Tsichlis, and J. Coffin, in preparation).

4. It has been recently shown by W. Hayward that in the majority of ALV induced B cell lymphomas in chickens, the transforming gene that becomes activated is the endogenous gene of the MC29 virus (myc). We have examined the expression of this gene in the NTRE7 induced chicken carcinomas and fibrosarcomas, as well as in the MoMuLV induced rat thymomas that we are studying. In both of these systems we found no evidence of expression of the myc gene. The high frequency by which myc is expressed in B cell lymphoma and the absence of its expression in chicken fibrosarcomas and carcinomas as well as in rat thymomas indicates tissue specificity for the activation of transforming genes during the process of viral oncogenesis.

In order to identify the transforming gene whose activation is responsible for each kind of tumor we are studying, we are following different approaches. (i) Identify by restriction analysis and Southern blotting of tumor derived high molecular weight DNA, junction fragments between virus and cell DNA that appear in many tumors. These fragments are ultimately cloned and tested. (ii) Try to isolate transforming viruses by infecting NIH 3T3 cells with tumor extracts. (iii) Screening of transcripts with various available transforming gene probes. These studies are described in more detail in the report by Dr. L. F. Hu.

5. In collaboration with Dr. T. Papas the avian myelocytomatosis virus (MC29) has been cloned from the integrated provirus of the Q5 line of MC29 transformed nonproducer quail cells. We have shown that the genome cloned is biologically active, as it is capable of inducing foci of transformation with high frequency in NIH 3T3 cells. The fact that the transformed cells are induced by the transfected MC29 genome has been shown in DNA-DNA hybridization experiments. Further analysis of these clones is now in progress.

Significance to Biomedical Research and the Program of the Institute:

Our studies have already provided considerable insight into the mechanism of virus induced oncogenesis. This understanding provides now a conceptual framework for study of nonviral tumors in animals including humans.

Proposed Course:

1. In experiments that will be done in collaboration with M. Ostrowski and G. Hager, we plan to examine the levels of the integrated viral DNA as well as viral RNA in cells infected with RAVO or NTRE7 both in the acute and the chronic stage of the infection. Subcloning of the clones we have generated into bovine papilloma virus (BPV) might allow us to study U_3 initiated transcription in vivo using nonintegrated molecules. This is probably important in order to control for the role of sequences adjacent to the integrated provirus on transcription. Finally, in vitro transcription studies using the cloned molecules available could provide clues regarding the biological differences conferred upon these two viruses by their different large terminal repeats.

2. We plan to identify the transforming genes defined by the U_3 initiated cellular transcripts in the NTRE7 induced chicken fibrosarcomas and carcinomas as well as in the MoMuLV induced rat thymomas. These genes will be studied in terms of their organization, natural distribution and expression in normal tissues and in viral and nonviral tumors.

3. Regarding the role of the region adjacent to U_3 in oncogenesis, we plan to generate recombinants between endogenous and exogenous avian retroviruses carrying the U_3^{end} allele. Study of the oncogenic potential of these viruses will provide a fine analysis of the genetics of viral oncogenesis.

4. In collaboration with Dr. Harriet Robinson, we will study the early events in viral oncogenesis that could potentially be responsible for the difference observed between NTRE7 and other exogenous viruses.

5. We will concentrate on the protein coded by the MC29 transforming gene. As a first step in this process we will try to generate antibodies directed against the MC29 specific portion of the MC29-gag polyprotein. These antibodies will be very valuable tools for future studies.

Publications:

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tsichlis, P. N., and Papas, T. S.: Molecular cloning of avian myelocytomatosis virus (MC29) transforming sequences. Proc. Natl. Acad. Sci. U.S.A. 78: 1518-1522, 1981.

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Spyropoulos, D., Tsichlis, P. N., and Papas, T. S.: The transforming sequences of avian myelocytomatosis virus (MC29). Differentiation and function of hematopoietic cell surfaces. In Gallo, R. and Fox, F. (Eds.): Proc. ICN-UCLA Symposia on Molecular and Cellular Biology. Vol 19, 1981, in press.

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Spyropoulos, D., Tsichlis, P. N., and Papas, T. S.: The transforming sequences of MC29. J. Supram. Structure, in press.

Robinson, H. L., Blais, B. M., Gagnon, G. M., Tsichlis, P. N. and Coffin, J. M.: U_3 encodes the oncogenic potential of avian leukosis viruses sequences 5' of U_3 target avian leukosis viruses towards lymphomagenesis. Proc. Natl. Acad. Sci. U.S.A. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05118-02 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Analysis of Transcriptional Regulation of Murine Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.C. Ostrowski	Staff Fellow	LTVG	NCI
OTHERS:	D.S. Berard	Microbiologist	LTVG	NCI
	G.L. Hager	Head, Viral Immunogenetics Section	LTVG	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Tumor Virus Genetics

SECTION
Viral Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro transcription of molecularly cloned murine retroviruses has been examined. Two type C retroviruses, AKR murine leukemia virus and Harvey sarcoma virus, were found to contain active RNA polymerase II promoters within the viral long terminal repeat sequences located at either end of the virus. Therefore, viral promoters can initiate transcription of adjacent cell sequences in vitro. In addition to the promoter within the LTR, Ha-MuSV contains a RNA polymerase III promoter. The 5' end of this transcript is approximately coincident with the viral transforming gene. This transcript has also been identified in Ha-MuSV transformed NIH 3T3 cells. Unlike type C retrovirus, the LTR promoter of the type B retrovirus, mouse mammary tumor virus, is inactive in our cell-free transcription system. Since initiation of MMTV transcription is regulated by glucocorticoid steroid hormones, it is likely the presence of hormone-receptor complex, as well as chromatin components that interact with this complex, are necessary for in vitro transcription of MMTV. Experiments designed to test this possibility and also to pinpoint the hormone target site within MMTV are in progress.

Project Description

Objectives:

(1) Molecularly cloned proviruses are to be tested as templates in a cell-free transcription system. 5' ends of *in vitro* transcripts will be determined and compared to 5' ends of cellular viral RNA.

(2) Hybrids between viral LTRs, viral structural genes and bovine papillomavirus will be constructed by molecular cloning. These hybrids will be transfected into susceptible cells, and resulting transformed cells will be analyzed. Attention will be focused on the state of the newly acquired DNA and the RNA initiated by retrovirus promoters.

(3) Episomal hybrid DNA will be isolated as nucleoprotein particles that will be used as template for *in vitro* transcription. Protein components of these nucleoprotein particles will be analyzed.

(4) Deletion mutants of retroviral LTR's will be made and combined in hybrids such as those described above. The behavior of these cloned mutants will then be studied as described above and compared to parental hybrids. These experiments should allow a determination of the LTR sequences involved in initiation of transcription and, in the case of MMTV, hormone regulation.

Methods Employed:

(1) Transcription extracts are prepared by published procedures. Molecular clones of retroviral DNA, particularly regions involved in transcription regulation, are isolated and characterized by restriction endonuclease mapping.

(2) The initiation sites for retrovirus transcription are determined by characterizing "run-off" products from viral DNA subclones. These sites are established more precisely by S1 and mung bean nuclease mapping of the RNA 5' ends.

(3) Molecular hybrids are constructed from purified restriction fragments of cloned viruses utilizing pBR322 as a bacterial cloning vector, and BPV or SV40 derivatives as eukaryotic vectors.

(4) DNA from tissue culture cells transformed with the molecular chimeras is analyzed by Southern blot analysis. Viral-specific transcripts are characterized by Northern blot analysis and nuclease mapping.

(5) Cells transformed with episomal replicating vectors containing viral regulatory sequences will be identified by Hirt fractionation. Nucleoprotein complexes of these episomal elements will be isolated by virtue of their relative small molecular size. These complexes will be utilized as substrates for *in vitro* transcription studies, and will be characterized for elements involved in transcription regulation.

(6) Deletion mutants are constructed by exonuclease treatment of appropriate cloned DNA's using pBR322 as vector.

Major Findings:

In vitro transcription of molecularly cloned type C retroviruses has demonstrated that these viruses contain active promoters within their LTRs. These promoters do not require information contained in adjacent host cell sequences in order to function *in vitro*. Mapping of the 5' ends of *in vitro* and *in vivo* viral RNAs indicate that the *in vitro* promoters are also active intracellularly. Integrated retroviruses contain two copies of the LTR located at either end of integrated provirus. *In vitro*, both copies of LTR are found to contain active promoters that initiate transcription at identical rates. Thus, *in vitro*, retroviruses are capable of expressing host cell sequences located downstream of the 3' LTR. Recent findings by other investigators suggest that such "promoter insertion" activity is important in oncogenic activity of some retroviruses. In addition to the pol II promoter in the LTR, the acute transforming virus Ha-MuSV was found to contain an -amanitin-resistant promoter. Transcripts initiated by this promoter can be terminated to yield a 340 nucleotide product. The α-amanitin resistance and termination of this *in vitro* product suggest that pol III is responsible for its transcription. We have also identified the existance of this transcript in tissue culture cells transformed by Ha-MuSV p-21 gene product and are presently conducting experiments to determine drug resistance. We also plan to determine whether this promoter is contained within molecular clones of endogenous rat p-21 genes. While the biological significance of this pol III promoter is presently unknown, the fact that the 5' end of this transcript is coincident with the 5' end of the Ha-MuSV transformation-specific gene is of potential interest.

Currently, hybrids between viral LTRs and bovine papillomavirus have been cloned in bacteria and transfected in tissue culture cells. Bovine papillomavirus provides a unique cloning vector for introducing sequences of interest into eukaryotic cells because it replicates as an episome to 50-100 copies per cell. Further studies utilizing this system should prove very valuable in elucidating transcriptional control elements of retroviruses.

Significance to Biomedical Research and the Program of the Institute:

Murine tumor viruses have served as model systems for studying the etiology of neoplastic diseases. It is now clear that genes homologous to the onc genes of these viruses are present in normal vertebrate cells, and that in some cases these cellular genes can be activated to produce transformation. The mechanisms by which the expression of these genes are regulated becomes of obvious importance to the understanding and ultimate control of oncogenic disease. Our efforts are aimed at the ultimate description in molecular terms of these regulatory mechanisms.

Proposed Course:

Bovine papillomavirus-retrovirus hybrids will be constructed. Regulation of transcription by LTRs will be studied utilizing *in vitro* mutagenic approaches. The hybrids should also provide the basis for reconstituting transcriptional control *in vitro* since hybrid DNA can be isolated from transfected cells in association with potentially important cellular components.

Publications:

Ostrowski, M. C., Hager, G. L.: *In vitro* transcription of murine type C retroviruses. *J. Supramolecular Structure and Cellular Biochem.* Suppl. 5, 434, 1981.

Ostrowski, M. C., Berard, D., and Hager, G. L.: Specific transcriptional initiation *in vitro* on murine type C retrovirus promoters. *Proc. Natl. Acad. Sci. USA* (in press) 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05119-02 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and Examination of Erythroleukemia Cell Lines from Mice Infected with Friend Murine Leukemia Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. I. Oliff	Research Associate	LTVG	NCI
OTHER:	D. Linemeyer	Staff Fellow	LTVG	NCI
	S. Ruscetti	Senior Staff Fellow	LTVG	NCI
	E. M. Scolnick	Chief	LTVG	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Friend murine leukemia virus (F-MuLV) is a retrovirus obtained from stocks of Friend virus complex (F-MuLV plus spleen focus-forming virus). Each of these viruses can produce a fatal erythroleukemia upon injection into certain strains of mice. However, the erythroleukemia cell lines obtained from these animals have always been isolated from mice infected with both of these agents. We have developed a technique which enables us to reproducibly obtain erythroleukemia cell lines from mice infected with F-MuLV in the absence of any spleen focus-forming virus. These cell lines can be transplanted into syngeneic mice where they grow and produce a fatal leukemia. This technique involves the bi-weekly transfusion of RBCs into diseased mice infected with F-MuLV. These mice normally die from a profound anemia associated with the F-MuLV infection. However, the transfused mice live for longer periods. During this time they develop transplantable leukemic cells which we can isolate and grow in vitro. These tissue culture lines are erythroid in nature as evidenced by positive staining with an anti-spectrin antibody. They cause a donor cell leukemia in transplant recipients as shown by sex chromosome analysis. Future work will analyze the changes in the DNA of these leukemia cells which occur when the cells become malignant.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. Isolation of erythroleukemia cells from mice infected with F-MuLV in the absence of spleen focus-forming virus.

2. Growth of these erythroleukemia cells in vitro.

3. Identification of the site(s) of insertion of the F-MuLV provirus into the host cell genome in these transplantable erythroleukemia cells and in the non-transplantable erythroleukemia cells obtained from mice infected with F-MuLV, but not transfused with red blood cells.

4. Identification of the RNA transcripts being produced by these transplantable erythroleukemia cells and those being produced by the non-transplantable erythroleukemia cells.

5. Assessment of whether the RNA transcripts from these cells correspond to the transformation gene sequences associated with the sarcoma inducing RNA tumor viruses.

Methods Employed:

1. Isolation of nucleic acids by phenol extraction of freshly grown cell lines which have been single cell cloned to insure the mono-clonal nature of the resulting DNA preparation. In the case of RNAs, purification of the messenger RNA species from the total cellular RNA by means of poly-A selection using oligo-dT columns.

2. Restriction enzyme digestion of the DNAs obtained from these cells with a number of enzymes (e.g. HindIII, EcoRI, and SalI) in order to generate DNA fragments of suitable sizes for analysis upon agarose gels.

3. Agarose gel electrophoresis, and Southern blot transfer and hybridization with P^{32} labeled cDNA probes for the DNA blots. Agarose-formaldehyde gel electrophoresis using formamide in the sample buffers for the RNA gel electrophoresis.

4. Molecular cloning in plasmid vectors (pBR322) of any restriction fragments which appear to be constant in the transplantable cell lines, or of any fragments which appear homologous to the known sarcoma gene products. These fragments will be isolated by gel electrophoresis and electroelution, followed by ethanol precipitation. Molecular cloning then proceeds via ligation of the appropriate molecules using T_4 DNA ligase and transformation of appropriate bacterial hosts using $CaCl_2$ and heat shock.

5. $CaCl_2$ precipitation of DNA to transfect mouse fibroblasts. DNA preparations are prepared in Hepes buffer and mixed with $CaCl_2$ in order to generate a microscopic precipitate which is taken up by the fibroblast monolayer and incorporated into the genome of the recipient cells.

Major Findings:

1. F-MuLV alone is capable of inducing transplantable erythroleukemia cells in susceptible mice. In constrast to the previously studied murine erythroleukemia cell lines (MELC) which all contained SFFV in addition to F-MuLV, our cell lines contain F-MuLV alone. These cells also do not express the major gene product from the pathogenic region of the SFFV genome, i.e., gp52. Therefore, it is no longer clear if SFFV alone has had any effect upon the generation of the previously reported MELCs, while it is apparent that F-MuLV alone can be used to induce the formation of these cells.

2. These cells can be grown as subcutaneous tumors upon injection into syngeneic mice. The erythroleukemia cells which form the predominant cell population in diseased mice infected with either F-MuLV or SFFV cannot be transplanted into syngeneic animals. Only after these tumor cells undergo an additional "transformation" can they then be explanted into tissue culture and/or grown *in vivo* as subcutaneous tumors. This second stage of malignancy has been described previously in Friend erythroleukemia cells. However, these characteristics were also thought to possibly be related to the effect(s) of SFFV upon the phenotype of the MELCs. Moreover, the growth of these cells *in vitro* allows us to obtain large quantities of pure erythroleukemia cell nucleic acids for analysis without the contamination associated with tumor cells obtained directly from diseased animals.

3. The RNA messages produced in these cells do not appear to correspond to any of the known retroviral sarcoma genes. The messenger RNAs produced by these *in vitro* MELC lines have been analyzed by agarose-formaldehyde gel electrophoresis and blot transfer to nitrocellulose paper for hybridization. Radiolabeled probes specific for the "sarc" gene sequences from eight different retroviral sarcoma viruses have been tested against these messenger RNA preparations. As yet none of these probes have cross hybridized to any of the F-MuLV induced MELCs messenger RNAs.

Significance to Biomedical Research and the Program of the Institute:

The establishment of a model system where a single virus can produce both a fatal erythroleukemia which is not transplantable and an erythroproliferative syndrome, which is both transplantable and explantable into tissue culture, allows us to examine the changes in the host cell genome which are responsible for these differences in phenotype. The first instance is much more akin to an uncontrolled hyperplastic response of erythroid precusors than the second disease syndrome which behaves more like a malignant process. Thus, we have two very similar cell types which contain the same foreign DNA, but which behave in very different ways. Since this foreign DNA has been previously very well characterized by molecular cloning, it can now serve as a probe for the nucleic acid differences between these types of erythroleukemia cells. Hopefully, this information will indicate why the transplantable erythroleukemia cells have become malignant.

Proposed Course:

1. Extraction of the high molecular weight nuclear DNA from several of the *in vitro* transplantable erythroleukemia cell lines. Extraction of the same fraction of DNA from the non-transplantable *in vivo* erythroleukemia cells.

2. Isolation of the total cellular RNA from these same cells, followed by purification of the messenger RNA fraction from the total cellular RNA by means of an oligo-dT column.

3. Analysis of these nucleic acids by means of agarose gel electrophoresis and transfer to nitrocellulose filter paper followed by hybridization to specific radiolabeled DNA probes. Comparison of the nucleic acids from the transplantable versus the non-transplantable erythroleukemia cells by means of these specific probes.

4. Subgenomic DNA fragments corresponding to the pathogenic gene(s) of F-MuLV and the transforming gene from a number of sarcoma inducing retroviruses have already been prepared in a number of laboratories. These fragments will be "nick-translated" to generate the specific probes mentioned above. In this manner the relationship, if any, between the manifestation of transplantability among these cell lines and the presence and/or expression of known transforming sequences will be explored.

5. Transfection of the high molecular weight DNA from these *in vitro* cell lines and from the non-transplantable leukemia cells into NIH 3T3 fibroblasts and into bone marrow precusors growing *in vitro* in the Dexter bone marrow culture system. Experiments of this nature will demonstrate if any qualitative changes have occurred in the DNAs of the transplantable cell lines which now render them capable of transforming other cells without the assistance of specific viral vectors.

Publications:

Oliff, A., Linemeyer, D., Ruscetti, S., Lowe, R., Lowy, D. R., and Scolnick, E.: A subgenomic fragment of molecularly cloned Friend murine leukemia virus (F-MuLV) DNA contains the gene(s) responsible for F-MuLV induced disease. *J. Virol.* 35: 924-936, 1980.

Scolnick, E. M., Oliff, A., Linemeyer, D., and Ruscetti S.: Molecular genetics and cell culture assays for helper-independent and replication defective components of the Friend virus complex. In Fields, B., Jaenisch, R., and Fox, C. (Eds.): *Animal Virus Genetics*. New York, Academic Press, 1980, pp. 425-441.

Chattopadhyay, S. K., Oliff, A., Linemeyer, D., Lander, M., and Lowy, D.: Genomes of amphotropic and ecotropic murine leukemia viruses isolated from wild mice. *Virology* (in press)

Oliff, A., Ruscetti, S., Douglass, E., and Scolnick, E.: Isolation of transplantable erythroleukemia cells from mice infected with helper-independent Friend murine leukemia virus. *Blood* August, 1981 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05120-02 LTVG

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of the Genome Structure and Organization of Avian Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Lautenberger	Expert	LTVG	NCI
OTHER:	T. S. Papas	Head, Carcinogenesis Regulation Section	LTVG	NCI
	P. Tsichlis	Expert	LTVG	NCI
	J. P. Bader	Head, Cellular Transformation Section	LTVG	NCI

COOPERATING UNITS

C. Garon	LBV, NIAID
J. Chirikjian	Biochemistry Dept., Georgetown University
K. Rushlow	Biochemistry Dept., Georgetown University
R. Feldmann	CCB, DCRT, NCI
P. Duesberg	Dept. of Mol. Biol., University of California, Berkeley

COOPERATING UNITS (if any)

(see above)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Avian myelocytomatosis virus (MC29), a defective acute leukemia virus, has a broad oncogenic spectrum in vivo and transforms fibroblasts and hematopoietic target cells in vitro. We have used recombinant DNA technology to isolate and characterize the sequences which are essential in the transformation process. Integrated MC29 proviral DNA was isolated from a library of recombinant phage containing DNA from the MC29-transformed nonproducer quail cell line Q5. The cloned DNA was analyzed by Southern blotting of restriction endonuclease digests and by electron microscopic visualization of R-loops formed between the cloned DNA and MC29 or helper virus RNA. It was found that the 9.2 kb cloned DNA insert contains approximately 4 kb of viral sequences and 5.2 kb of quail cellular sequences. The viral sequences contain all of the MC29-specific sequences and 5' helper related sequences as well as part of the envelope region. The size of the cloned EcoRI fragment is the same as that of the major band in EcoRI-cleaved Q5 DNA that hybridizes to viral sequences. Transfection of the cloned DNA into NIH 3T3 cells, revealed that the MC29-specific sequences are functional, in that they induce foci of transformed cells with high efficiency.

Project Description

Objectives:

The scope of this investigation is to determine the role of different portions of the genome of an avian retrovirus in cell type specific transformation. The avian myelocytomatosis virus, MC29, has been selected since it can transform fibroblasts in vitro. The techniques of molecular cloning, DNA sequence analysis, and site-specific mutagenesis will be used to implicate specific nucleotides in the transformation process. The location of these nucleotides should provide a better understanding of how this virus is able to induce a specific form of leukemia.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K followed by phenol-chloroform extraction. Preparation of vector DNA from phage λ derivatives by phenol extraction of CsCl banded phage.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose gels.

3. Preparation of cDNA probes using purified RSV or AMV(MAV) RNAs as template and fragmented calf thymus DNA or poly dT as primers. Probes also are prepared by nick translation of cloned DNA using E. coli DNA polymerase.

4. Preparation of nitrocelluose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of the cDNA probe to virus-related sequences on these filters.

5. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.

6. Isolation of phage from the libraries containing virus-related sequences by hybridization of cDNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.

7. Transfection of NIH 3T3 cells by exposing them to cloned viral DNA that had been co-precipitated with calcium phosphate.

8. R-loop and heteroduplex analysis of cloned DNA with viral RNA and cloned proviral DNA from other viruses.

9. Computer analysis of DNA sequences. The program Queen and Korn lists DNA sequences, locates restriction sites, translates sequences, finds repeated regions, finds self-complementary regions and compares a sequence with sequences of other proviral DNAs. The programs of R. Staden implemented on

the NIH DEC-10 perform similar functions and also allows one to concentrate sequences from individual gels into an overall sequence. A program written by Richard Feldmann displays the most probable secondary structure of a 200 base section of RNA. We have developed a program that allows us to use a Tektronics computer equipped with a digitizer to automatically read gels. The sequences from each individual gel can be transmitted to the NIH DEC-10 for use with the Staden sequence concatenation program.

10. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert and by the dideoxyribonucleotide method of Sanger and associates. For the latter method, the cloned DNA is recloned into a filamentous phage.

Major Findings:

1. A 9.2 kb EcoRI fragment that hybridizes to an AMV probe was isolated from a library of recombinant phage containing DNA from the MC29-transformed non-producer cell line Q5. This fragment was found to contain 4 kb of viral sequence and 5.2 kb of quail cellular sequences. The viral sequence contained the left large terminal redundancy, the 5' helper related ("gag") sequences, the MC29 specific sequences and a portion of the 3' helper-related ("env") sequences. The fragment had the same size as the major band in EcoRI-cleaved Q5 DNA that was found to hybridize to a viral probe.

2. The cloned DNA formed a forked structure with MC29 viral RNA under R-loop formation conditions. The absence of looped structures within the hybrid region is evidence for colinearity of cloned provirus sequences and viral RNA. We therefore conclude that there is no structural evidence for sequence rearrangement in the proviral sequences of the cloned DNA.

3. The cloned MC29 proviral DNA is capable of transforming NIH 3T3 cells to form foci with a frequency of approximately 1,000 foci per μg. Preliminary analysis indicates that cell lines from these foci contain viral sequences.

4. We have used the proviral DNA of the avian acute leukemia virus MC29 to screen chicken DNA library in phage λ for cellular sequences homologous to the viral mov sequence. A partial restriction map of the three overlapping cellular DNA inserts, which span a region of over 30 kb in length, has been determined. Heteroduplex analysis indicated that the cellular DNA contains two exons divided by a single intron. The exon sequences are colinear with their viral homologs.

5. The DNA sequence of the left LTR within the cloned MC29 proviral DNA is being determined. The U_3 region of MC29 is very similar to the U_3 region of Rous sarcoma virus, but is quite different from the sequence of the AMV LTR U_3 region determined by K. Rushlow.

BEST DOCUMENT AVAILABLE

Significance to Biomedical Research and the Program of the Institute:

MC29 is especially interesting in that it transforms specific target cells *in vivo* that constitute a very small fraction of the hematopoietic cell population. Since the virus can transform both fibroblasts and bone marrow cells *in vitro*, it should be possible to determine which viral sequences are required for transformation and whether transformation of bone marrow cells requires sequences in addition to those needed for fibroblast transformation. By comparison of the location of the transforming sequences with the MC29 genetic map, it should be possible to determine the role of the different virus-specified proteins in transformation. Understanding the molecular biology of this cell type specific transforming virus should provide general insight into the mechanism of the leukemic process.

Proposed Course:

1. The transforming regions of MC29 will be defined by further transfection experiments and by characterization of viral products within cells transformed by cloned viral DNA. NIH 3T3 cells will be transfected with specific restriction fragments. The overlaps between those fragments that form foci will define the necessary region. It will be of particular interest to know whether "gag" sequences are needed for transformation

   The nature of the viral DNA sequences within the transformed cells will be determined by the Southern procedure. The presence of viral sequences in bands not found in untransformed cells will confirm that transformation was mediated by the uptake of viral sequences. It will be of interest to determine if the transformed cells contain sequences related to the LTR and "gag" sequences in the cloned DNA.

   Expression of viral sequences will be studied by the Northern procedure. The cell RNA is electrophoresed in a denaturing gel and immobilized on a filter. Viral messages can be detected by hybridization to labeled cloned viral DNA.

   The proteins in these cells that contain viral sequences will be characterized by labeling the cells with ^{35}S-methionine and precipitating the viral proteins with antisera to AMV or ASV.

2. The DNA sequence of important regions within the cloned DNA will be determined. Such regions include the LTR, the area around the "gag" initiator codon and the MC29-specific sequences. These sequences should allow identification of control regions such as promoters, polyadenylation sites, and ribosome binding sites. The sequence of the MC29-specific region should reveal whether it codes for any proteins besides the 110,000 dalton protein identified in nonproducer cells.

BEST DOCUMENT AVAILABLE

Publications:

Lautenberger, J. A. Edgell, M. H., and Hutchison III, C. A.: The nucleotide sequence recognized by the BstEII restriction endonuclease. Gene 12: 171-174, 1980.

Lautenberger, J. A., White, C. T., Haigwood, N. L., Edgell, M. H., and Hutchison III, C. A.: The recognition site of type II restriction enzyme BglI is interrupted. Gene 9: 213-231, 1980.

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tsichlis, P. N., and Papas, T. S.: Molecular cloning of avian myelocytomatosis virus (MC29) transforming sequences. Proc. Natl. Acad. Sci. USA 78: 1518-1522, 1981.

Lautenberger, J. A., Shulz, R. A., Garon, C. F., Tsichlis, P. N., Spyropoulos, D. S., Pry, T. W., Rushlow, K. E., and Papas, T. S.: The transforming sequences of avian myelocytomatosis virus (MC29). J. Supramol. Struct. 5: 116, 1981.

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tsichlis, P. N., Spyropolous, D. S., Pry, T. W., Rushlow, K. E., and Papas, T. S.: The transforming sequences of avian myelocytomatosis virus (MC29). In Fox, C. F. (Ed.): ICN-UCLA Symposium on Differentiation and Function of Hematopoietic Cell Surfaces. New York, Alan R. Liss, 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05121-02 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of the Expression of Mouse Mammary Tumor Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. D. Johnson	Postdoctoral Fellow	LTVG	NCI
OTHER:	D. S. Berard	Microbiologist	LTVG	NCI
	R. G. Wolford	Microbiologist	LTVG	NCI
	G. L. Hager	Head, Viral Immunogenetics Section	LTVG	NCI

COOPERATING UNITS (if any)

L. O. Arthur   Biological Carcinogenesis Program   FCRC

LAB/BRANCH
Carcinogenesis Intramural Program, Laboratory of Tumor Virus Genetics

SECTION
Viral Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0

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☐ (a) HUMAN SUBJECTS   ☐ (b) HUMAN TISSUES   ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The site of mouse mammary tumor virus RNA transcription initiation has been determined in the presence and absence of glucocorticoid hormone (dexamethasone), and found to be invariant. Molecular clones of the Mtv-1 locus (responsible for mammary tumor induction in C3Hf mice) have been characterized. Transfection of XC/TK$^-$ cells with ligated Mtv-1 DNA and TK DNA gives rise to TK$^+$ transfectants that express the Mtv-1 cloned DNA, both as glucocorticoid-regulated RNA and hormonally-induced gp52 env antigen. Sequencing of the Mtv-1 5' long terminal redundancy (LTR) identifies one region (~100 nucleotides) of major rearrangement compared with the exogenous C3H-S LTR; this region is presumably not involved in the hormone target area.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

(1) Structural analyses of full-length molecular clones of endogenous, Mtv-1 proviral DNA by restriction endonuclease mapping and sequence determination.

(2) Analysis of hormone-inducible transcription initiation sites for RNA from MMTV-induced tumor cell lines and heterologous MMTV-infected HTC cells having different induction phenotypes.

(3) Establishment of the potential of the full-length molecular clone of endogenous Mtv-1 proviral DNA to express normal MMTV transcripts and gene products and to manifest the hormone-responsive induction phenotype.

(4) Elucidation of the molecular mechanisms involved in hormone regulation utilizing the Mtv-1 cloned DNA.

(5) Determination of the oncogenic potential of the Mtv-1 cloned DNA and investigation of mechanisms of transformation using these reagents.

Methods Employed:

(1) Subcloning of the Mtv-1 DNA in bacteriophage lambda and plasmid pBR322 will be performed. Fine restriction endonuclease maps and sequencing of the regulatory promoter and regulatory regions of Mtv-1 cloned DNA will be carried out.

(2) Utilizing the S1 nuclease or mung bean nuclease mapping techniques, probes available from molecular clones of MMTV will be utilized to identify the specific start site for transcription in the absence and presence of glucocorticoid hormones. The region of the probe protected by RNA will be analyzed at the nucleotide level by employing probe-sequencing reactions as marker.

(3) The biological activity of the cloned Unit V proviral DNA will be determined. Aspects such as RNA synthesis, protein synthesis, transcription start site, and transmissibility will be analyzed.

(4) Co-transfections using cloned viral DNA and thymidine kinase gene DNA will be performed using the calcium phosphate technique and HAT selective media.

Major Findings:

Transcription regulation of mouse mammary tumor virus by glucocorticoids has been examined in detail. RNA from MMTV virions and total cellular RNAs from the tumor-derived 34i cl 101 cell line and from HTC cell lines infected with virus and having low proviral DNA copy number, as well as different induction phenotypes, all have the same transcription initiation site in the absence and presence of dexamethazone.

Thus, hormones do not enhance viral RNA synthesis by altering the site of transcription initiation but rather through some new interaction at the same site.

Knowledge of the exact initiation nucleotide for viral RNA synthesis and the site of polyadenylation permits the definition of the viral RNA repeat for MMTV as 14 bases.

Restriction mapping of Mtv-1 and other proviral units has elucidated restriction sites specific for the endogenous proviral DNAs of the C3H mouse.

A complete, endogenous, proviral DNA of MMTV has been utilized for beginning studies in the area of gene regulation. This DNA is the only full-length DNA copy identified in molecular cloning experiments in this laboratory. This proviral DNA represents the Mtv-1 locus of the C3Hf mouse. This genetic locus is associated with the formation of late tumors in this mouse strain. The product of linkage of MMTV DNA to the thymidine kinase gene was transfected onto a non-murine thymidine kinase negative cell line (XC TK^-). This cell line was used since it contains no background of MMTV genetic information. After selection for the TK^+ phenotype in HAT medium, individual foci were expanded.

Hormone stimulated transcription is found in these transfectants. Analysis of cellular RNA indicates a 10-fold increase in the envelope glycoprotein mRNA of MMTV. The cloned Mtv-1 DNA therefore represents a biologically competent copy of the provirus. The transcription start site for total MMTV RNA synthesis in these transfectants is the same as that found for exogenous virus. Thus, there is no difference in hormone control of transcription initiation when comparing the endogenous and exogenous DNAs.

The transfected cell clones also make viral proteins. Using RIA techniques, hormone-inducible gp52 is found in cellular extracts. Immune precipitation studies are underway to identify specific proteins. A small amount of gp52 is found by RIA in the culture fluid.

Sequence analysis of the long terminal redundancy, host flanking regions and unique viral information is in progress. Comparison of endogenous Mtv-1 (Unit V) DNA to that of endogenous Unit II DNA and exogenous C3H-S (Unit IV) DNA indicates minor base substitutions within the LTRs. However, Unit V LTR has a major rearrangement near the 3' end of the open reading frame found in both Units II and IV. Within the unique sequences immediately downstream from the left LTR, a potential splice junction for the MMTV leader has been identified. Comparison of viral LTR, viral unique and host sequences for both LTRs have identified a 6 bp host repeat immediately adjacent to the viral DNA. A potential analogy exists between the integration model for TN10 and MMTV Unit V.

Significance to Biomedical Research and the Program of the Institute:

Mouse mammary tumor virus has served as a model system not only for studying the etiology of a mammary neoplasia induced by an RNA tumor virus, but also for investigating the mechanisms underlying hormonal regulation of eukaryotic gene expression. The experimental program undertaken in this project is directed at providing a molecular framework within which both the oncogenic activity of this virus and the control processes that affect its regulation can be understood. A major goal of this program has now been achieved, in that we have generated what we believe is an accurate, non-rearranged molecular clone of the oncogenic, hormonally regulated Mtv-1 locus.

Proposed Course:

Given the ability to distinguish between molecular clones of C3H-S (Unit IV), Unit II and Mtv-1 (Unit V) DNA, the C3Hf virus will be examined for its identity to any of these proviral units.

When specific antibody to the protein (LTR-ORF) coded for by the LTR becomes available, the cells transfected with Mtv-1 DNA will be tested.

The presence of gp52 in the culture fluid of cells transfected with Mtv-1 proviral DNA suggests the possibility of mature viral production. Transmission studies to confirm or negate this possibility will be performed. If negative, the cloned Mtv-1 DNA will be transfected into other heterologous cells known to produce mature virions. The potential biological importance of the cloned DNA for tumor formation makes it important to test the oncogenicity of released virus in an animal system.

The identification of a cloned glucocorticoid sensitive DNA molecule permits the dissection of hormone regulation of transcription. Deletion mutants of the cloned proviral DNA will be made. The first set will be block deletions of the proviral DNA upstream from the transcription initiation start site. After linkage to TK, DNA and transfection foci will be tested for hormone induction of MMTV transcripts to locate potential hormone regulatory regions.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05122-02 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Transforming Sequences of Rat-derived Sarcoma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Ellis	Staff Fellow	LTVG NCI
OTHER:	D. (Defeo) Jones	Microbiologist	LTVG NCI
	E. M. Scolnick	Chief	LTVG NCI

COOPERATING UNITS (if any)

H. A. Young	Biological Carcinogenesis Program, Frederick Cancer Research Center
M. A. Gonda	Biological Carcinogenesis Program, Frederick Cancer Research Center
D. R. Lowy	Dermatology Branch, NCI

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The transforming src genes of Harvey (Ha-) and Kirsten murine sarcoma virus (Ki-MuSV) encode p21. While these viruses encode highly related p21 polypeptides, their src genes are derived from cellular sarc genes which are distinct in all species studied. We have cloned two rat cellular sarc genes related to Ha-MuSV src. One gene is colinear with viral src while the other has three introns in its coding sequence. Nevertheless, both genes are capable of transforming fibroblasts and inducing high levels of p21 expression following DNA transfection. This project has involved recombinant DNA technology and other biochemical analyses.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The src gene of Ha-MuSV was defined and compared to that of Ki-MuSV src. Ha-MuSV src was used as a probe for related rat cellular sequences in a recombinant DNA "library." Rat sarc genes were subcloned in pBR322 and their structure was compared with Ha-MuSV src. The two rat cellular genes were used to transform cultured fibroblasts and these cells were tested for the p21 translational product of src.

Methods Employed:

1) Transfection of DNA by the calcium phosphate precipitation procedure. DNA molecules, which have been cloned in either phage or plasmid vectors, are purified as described in 4. Molecules sometimes are ligated using T4 DNA ligase. The DNA preparation is precipitated by calcium phosphate and transfected to NIH 3T3 cells. Transformed cell foci are isolated using a cloning cylinder and grown for protein and nucleic acid analysis. The transformation-specific viral p21 protein is detected by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. Cellular RNA is isolated and analyzed by agarose gel electrophoresis, Northern blotting, and hybridization with [^{32}P]-DNA probes. Cellular DNA is isolated and analyzed as described in 2.

2) Analysis of DNA by restriction endonuclease mapping and Southern blot hybridization. Cloned DNA and high molecular weight eukaryotic DNA is digested by restriction endonucleases at 2-10 units/μg for 2-16 hours. Enzymes are used either singly, doubly, or consecutively. DNA preparations are resolved by agarose gel electrophoresis in the presence of 0.0001% ethidium bromide. Restriction maps are constructed for cloned DNA molecules based upon overlap analysis of visual DNA bands. Homology of portions of cloned molecules and of high molecular weight DNA fragments is assessed following Southern blot hybridization analysis using [^{32}P]-DNA probes.

3) Molecular cloning of viral DNA in the plasmid vector pBR322 and in the bacteriophage vector λ. Restriction endonuclease maps are defined for either cloned or uncloned DNA preparations. Fragments with suitable termini are purified by gel electrophoresis, electroelution, DEAE-23 column chromatography, and EZOH precipitation. Fragments can be cloned directly at "cloning sites" in pBR322 (PstI, EcoRI, SalI, HindIII, BamHI), λgtWESλ·B (EcoRI, SacI), or Charon 4A (EcoRI). Alternatively, fragments with other termini are made flush-ended using PolI Klenow, altered by ligation with oligonucleotide linkers, and cloned at the relevant vector sites. Colonies are screened using the Hogness-Grunstein procedure.

4) Purification of DNA from phage, plasmid-infected bacteria, and eukaryotic cells. Cloned phage are grown in bacterial strain LE392 following infection at MOI = 0.05-0.10. After O.N. propagation, bacteria are disrupted by

chloroform, phage precipitated by polyethylene glycol and purified by cesium chloride density gradient centrifugation, and DNA purified following protease K digestions and phenol extraction. Cloned plasmids are propagated in bacterial strain RRI and amplified by chloramphenicol addition. Bacteria are lysed by SDS plus lysozyme, and plasmid DNA is purified from the Hirt supernatant by phenol extraction followed by A50M column chromatography. High molecular weight eukaryotic DNA is purified following cellular disruption by SDS + protease, RNase digestion, and repeated phenol extractions.

Major Findings:

1) The *src* genes of Ha-MuSV and Ki-MuSV are evolutionarily conserved and distinct from one another. Specific DNA probes were cloned from the Ha-MuSV and Ki-MuSV *src* genes. These probes were hybridized to Southern blots of digested high molecular weight DNAs from a variety of species. The respective *sarc* genes are broadly conserved. The different sized fragments hybridized by the two probes in each species demonstrate the distinction of Ha-*sarc* and Ki-*sarc* genes which number 1-2 in each species tested. In combining the results of heteroduplexing and crosshybridizations of Ha-*src* and Ki-*src* clones under varying degrees of stringency, we conclude that each *src* gene has two domains, one 10-15% divergent between the two *src* genes, the other >25% divergent.

2) One cellular *sarc* gene cloned from rat genomic DNA is colinear with Ha-MuSV *src*. This gene (Ha-*sarc* I), which corresponds to the 13 kbp *Eco*RI digest band in rat DNA, was cloned at the *Eco*RI sites of vector Charon 4A. A 2.2 kbp *Eco*RI-*Bam*HI subclone in pBR322 was prepared. Restriction mapping, hybridization, and heteroduplex analysis revealed complete colinearity with Ha-MuSV *src*. Ligation of an LTR-containing clone of Ha-MuSV to Ha-*sarc* I resulted in cell transformation and expression of a p21 indistinguishable from Ha-MuSV p21.

3) A second cellular *sarc* gene cloned from rat genomic DNA has 3 introns in its coding sequence. The total length of the 4 exons is the complete size of Ha-MuSV *src* as previously estimated. This gene (Ha-*sarc* II), which corresponds to the 20 kbp *Eco*RI digest band in rat DNA, was cloned at the *Eco*RI sites of vector Charon 4A. A 5.4 kbp *Eco*RI-*Bam*HI subclone in pBR322 was prepared. Restriction mapping, hybridization, and heteroduplex analysis revealed 3 introns in its coding sequence as well as some restriction site mismatch with Ha-MuSV. Ligation of Ha-MuSV LTR to Ha-*sarc* II resulted in cell transformation and expression of a p21 distinct from the p21 of Ha-MuSV and Ha-*sarc* I.

4) Both cellular genes, following ligation to LTR promoter sequences, induce cellular transformation and p21 expression following DNA transfection. These observations suggest that the mere elevation in the level of expression of a normal cellular protein is sufficient to induce cellular transformation.

5) Ha-MuSV and Ki-MuSV likely were formed by a minimum of two recombinational events. The *src* genes of Ha-MuSV and Ki-MuSV are flanked by "30S" sequences. "30S" represents genes for replication-defective rat retroviruses whose major transcriptional product resolves as a "30S" RNA by sucrose density gradient

centrifugation. Since neither Ha-sarc I nor Ha-sarc II is flanked in rat DNA by "30S" and since the viral LTR is derived from murine leukemia viruses, Ha-MuSV and Ki-MuSV must have been formed by at least two recombinational events at either the DNA or RNA level.

Significance to Biomedical Research and the Program of the Institute:

The ability of the cellular Ha-MuSV related sarc genes to induce cellular transformation suggests that elevated levels of a normal cellular gene are sufficient to induce dramatic changes in cellular physiology. In concert with studies of endogenous Ki-MuSV related sarc genes, the entire family of rat-derived sarc genes can be isolated, expressed *in vitro*, and studied with a view toward understanding the normal function of these genes.

Proposed Course:

1) Cloning endogenous Ki-MuSV related sarc genes. The Ki-MuSV src clone which we have developed will be used as a probe to isolate genes from rat and mouse genomic libraries.

2) Analysis of cells transformed by endogenous sarc genes. Cells will be studied with respect to DNA, RNA, and protein components related to the rat-derived transforming sequences.

3) Sequence analysis of src and cellular sarc genes and their flanking sequences. By the Maxam-Gilbert DNA sequencing technique, src and sarc genes will be sequenced in order to provide baseline information regarding the p21 gene family.

4) Analyzing the molecular processing of src and sarc RNA. Northern blots of normal cellular and viral mRNAs will be performed with different nucleic acid probes.

5) Studying the expression of these cellular genes in normal cells of different states of differentiation. RNA expression can be assessed both qualitatively and quantitatively. The DNase I sensitivity of sarc genes in intact chromatin can be assessed as a further paramter of gene expression.

Publications:

Chang, E.H., Maryak, J.M., Wei, C.-M., Shih, T.Y., Shober, R., Chang, H.L., Ellis, R.W., Hager, G.L., Scolnick, E.M., and Lowy, D.R.: Functional organization of the Harvey murine sarcoma virus genome. *J. Virol.* 35: 76-92, 1980.

Ellis, R. W., DeFeo, D., Maryak, J. M., Young, H. A., Shih, T. Y., Chang, E. H., Lowy, D. R., and Scolnick, E. M.: Dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma viruses. *J. Virol.* 36: 408-420, 1980.

Young, H. A., Gonda, M. A., DeFeo, D., Ellis, R. W., Nagashima, K., and Scolnick, E. M.: Heteroduplex analysis of cloned rat endogenous replication-defective (30S) retrovirus and Harvey murine sarcoma virus. Virology 107: 89-99, 1980.

Chang, E. H., Ellis, R. W., Scolnick, E. M., and Lowy, D. R.: Transformation by cloned Harvey murine sarcoma virus DNA: Efficiency increased by long terminal repeat DNA. Science 210: 1249-1251, 1980.

Scolnick, E. M., Shih, T. Y., Maryak, J., Ellis, R., Chang, E., and Lowy, D.: Guanine nucleotide binding activity of the src gene product of rat-derived murine sarcoma viruses. Ann. NY Acad. Sci. 354: 398-409, 1980.

DeFeo, D., Gonda, M. A., Young, H. A., Chang, E. H., Lowy, D. R., Scolnick, E. M., and Ellis, R. W: Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. USA 78: 0000-0000, 1981.

Ellis, R. W., DeFeo, D., Shih, T. Y., Gonda, M. A., Young, H., Tsuchida, N., Lowy, D. R., and Scolnick, E. M.: The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature, in press, 1981.

Gruss, P., Ellis, R. W., Shih, T. Y., Koenig, M., Scolnick, E. M., and Khoury, G.: SV40 recombinant molecules express the p21 transforming protein of Harvey murine sarcoma virus from an unspliced RNA. Nature, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05181-01 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

In Vitro Transformation of Hemopoietic Cells by Harvey and Kirsten Sarcoma Viruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. D. Hankins	Expert	LTVG NCI
OTHER:	E. M. Scolnick	Chief	LTVG NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Harvey and Kirsten-murine sarcoma viruses have previously been shown to transform fibroblastic cells in culture and type C virus pseudotypes of these viruses cause erythroleukemia in susceptible mice. We have developed a cell culture assay for quantitating the growth-promoting effect of Harvey and Kirsten viruses on erythroid cells. Murine hemopoietic cells were infected in vitro with Harvey or Kirsten sarcoma virus and then cultured in methylcellulose. Under these conditions, large colonies of erythroid cells form in the semi-solid culture media 6-8 days post-infection (Erythroid Bursts). The induction of erythroid bursts was not caused by the murine type C helper viruses used to pseudotype either Ha-MSV or Ki-MSV or by media from cells carrying the Ki-MSV and Ha-MSV genomes. Induction of the erythroid colonies is under genetic control at the Fv-1 susceptibility locus, but not at the Fv-2 susceptibility locus. A striking feature of the Harvey and Kirsten virus induced erythroid colonies was that they not only proliferated to large size but also differentiated along the erythroid lineage and synthesized hemoglobin. It is concluded that Ha-MSV and Ki-MSV can induce proliferation of erythroid precursor cells without apparently interfering with the differentiation program of the cells.

Project Description

Objectives:

The Harvey (Ha-MSV) and Kirsten (Ki-MSV) strains of murine sarcoma virus were isolated during the passage of murine type C helper-independent viruses through rats. Both viruses have been shown to be recombinant viruses with rat genetic information flanked by mouse retroviral sequences which represent the 5' and 3' ends of the viral genomes. In the original descriptions of the diseases produced by Ha-MSV and Ki-MSV, it was noted that the original pathogenic virus complexes induced erythroleukemia as well as inducing foci of transformed fibroblastic cells. Subsequently, a single biological agent in each case was shown to carry the ability to induce both foci on fibroblasts and erythroleukemia. Since the properties of the erythroleukemia cells have not been studied, it is not known whether these viruses directly transform hemopoietic cells or produce increased erythropoiesis secondary to transformation of other cell types. Nevertheless, the ability to cause a form of acute leukemia and induce foci classifies Ha-MuSV and Ki-MSV among many replication-defective retroviruses which cause transformation of fibroblasts and some form of hemopoietic malignancy.

During the past two years, others in this laboratory have defined the location of the gene in Ha-MSV and in Ki-MSV responsible for the transforming ability of the viruses. In addition, the product of the src gene for each virus as a 21,000 dalton protein which has an unusual affinity for guanine nucleotides, was characterized. These studies were possible because a quantitative assay for the transforming ability of the DNA forms of these viruses was available on NIH 3T3 cells and because large quantitites of viral DNA were available using molecularly cloned forms of the viruses. In order to study the leukemia-inducing potential of each virus, we sought to develop a quantitative assay which can measure in cell culture the hemopoietic effects of the viruses. To do this, we employed an approach successfully used earlier by the principal investigator to measure, in cell culture, the proliferative potential of another erythroleukemia-inducing retrovirus, the spleen focus-forming virus (SFFV).

Methods Employed:

Erythroid Transformation Assay. Spleen cells (from phenlhydrazine-treated CD2F1 mice) were incubated for 2 hours at 4°C with appropriately diluted virus sample, and after addition of semi-solid growth medium, the suspension was dispensed (0.5 ml/well) into Costar cluster dishes. The final suspension contained 30% fetal calf serum and 0.76% methylcellulose. Erythropoietin was added at a final concentration of 0.1 U/ml. The cultures were monitored daily through an inverted microscope. Quantitative assessment of the erythroid transformation was accomplished by scoring benzidine-positive erythroid bursts in stained clots prepared from the methylcellulose cultures, or by measuring ^{59}Fe incorporation into hemoglobin.

Major Findings:

1. When hemopoietic cells with erythroid precursors are infected *in vitro* with Ha-MSV or Ki-MSV, colonies of erythroid cells proliferate in semi-solid media. In media containing fetal calf serum, but no added EPO, a few erythroid colonies of moderate size appear but fail to undergo complete hemoglobinization, a late step in erythroid differentiation. In the presence of relatively low concentrations of added crude erythropoietin, the colonies reach much larger size and become distinctly red in color as they synthesize copious amounts of hemoglobin.

2. By using ^{59}Fe incorporation into heme in these colonies, a biochemical quantitation of the effects of virus and erythropoietin was possible as well as the visual counting of the macroscopic erythroid colonies.

3. The maximal level of both hemoglobin synthesis and burst formation was observed 6-8 days post-infection.

4. The number of erythroid bursts induced by Ki-MSV and Ha-MSV was directly related to virus dose and the number of cells plated.

5. Experiments with (i) various pseudotypes of Ha-MSV and Ki-MSV, (ii) sucrose gradient isopynically banded virus, and (iii) various methods of inactivating retrovirus strongly indicate that virus infection is required for the induction of growth of these erythroid colonies.

Significance to Biomedical Research and the Program of the Institute:

The significance of these studies is fourfold:

1. The demonstration of *in vitro* transformation of hemopoietic cells by Harvey/Kirsten viruses suggests that the erythroleukemogenic effects of these viruses result from direct infection of hemopoietic targets rather than arising secondary to other effects of the viruses.

2. By providing an *in vitro* system for direct analysis of the events which mediate the hemopoietic transformation(s), these studies should lead to a further increase of our understanding of the relationship of oncogenic proteins to both normal and pathologic development of hemopoietic precursors.

3. We found that Harvey- and Kirsten-transformed cells are still capable of differentiation and, in fact, are extremely sensitive to the action of the hormone, erythropoietin. Therefore, infection of hemopoietic tissues with these viruses to produce discrete clusters of erythroid cells provides a means of obtaining a homogeneous cell population of hormone sensitive precursors with which to perform studies on the mechanism of action of erythropoietin (and perhaps other glycoprotein hormones). It has not heretofore been possible, by conventional cell separation technologies, to ascertain such pure populations of erythropoietin sensitive cells.

4. The methodology developed in these studies--better cell culture methods, in vitro transformation, transfection of hemopoietic cells--is of general applicability and therefore should benefit hematology research in particular and other areas of cancer research in general.

Proposed Course:

1. Harvey- and Kirsten-transformed cells will be recovered from the semi-solid cultures. These cells will be tested for their (i) tumorigenicity in vivo, (ii) response to erythropoietin (mechanism of action studies), and (iii) ability to generate permanent (hormone responsive) tissue culture lines.

2. Experiments will be carried out to define the hemopoietic target cell specificity in an effort to ascertain whether viral infection or p21 expression is limited to erythroid cells in a particular stage of development.

3. We will attempt to find conditions for in vitro transfection of hemopoietic cells with high molecular weight DNA from Harvey-transformed cells or molecularly cloned DNA containing genetic information encoded by the Harvey/Kirsten viruses. Ultimately, this approach should permit direct analysis of the effects of specific virus (or cellular) genes on hemopoietic cells.

Publications:

Hankins, W. D., and Krantz, S. B.: Helper virus is not required for in vitro erythroid transformation of hematopoietic cells by Friend virus. Proc. Natl. Acad. Sci. USA 77: 5287-5291, 1980.

Hankins, W. D., and Troxler, D.: Polycythemia- and anemia-inducing erythroleukemia viruses exhibit differential erythroid transforming effect in vitro. Cell 22: 693-699, 1980.

Kost, T. A., Hankins, W. D., Glick, A., and Krantz, S. V.: Production of spleen focus-forming virus and murine leukemia virus by early erythroblasts after Friend virus infection. Cancer Res. 40: 1150, 1980.

Kost, T. A., Hankins, W. D., and Krantz, S. B.: Combined effect of Friend polycythemia virus and erythropoietin on erythroid burst formation in vitro. Exptl. Hematology 8: 248-258, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05182-01 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation of Cellular Revertants of Kirsten Virus Transformed NIH 3T3 Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Z. Selinger	Visiting Scientist	LTVG	NCI
OTHERS:	R. H. Bassin	Head, Viral Biochemistry Section	LTVG	NCI
	E. M. Scolnick	Chief	LTVG	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project is directed at development of conditions for isolation of cellular revertant of Kirsten virus transformed cells and characterization of their properties. The desired revertants should contain a rescuable wild-type Kirsten virus and produce the characteristic p21 protein as the src gene product. The system used is NIH 3T3 cell line that had been infected two consecutive times with Kirsten virus. Analysis of the DNA of this cell line showed that it contains two copies of the Kirsten genome. Condition for mutagenesis and selection procedure for isolation of revertant had been worked out. The cell revertants that had been isolated are currently being characterized.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The goal of this project is to isolate revertants that express normal growth properties and yet still contain the transforming Kirsten virus and synthesize the characteristic p21 src gene product. Conceivably such revertants are modified at a step in the transformation pathway initiated by the action of p21. Analysis of the molecular basis for the revertant phenotype is therefore an invaluable tool in studies of the mechanism of transformation by Kirsten virus.

Methods Employed:

(1) The frequency of cell revertants in the double transformed cell line is very low $< 10^{-4}$. To increase, the frequency of revertant mutagenesis is carried out with MNNG and 5 Azacytidine. Stock MNNG solution in ethanol is diluted with minimal essential medium without serum and buffered with 20 mM HEPES at pH 6.8. MNNG is added to cell culture freshly washed with the above buffer and incubation in presence of the drug is carried out for 3 h. 5 Azacytidine is added to cell culture in DMEM medium containing 10% fetal calf serum and incubation with the drug is continued for 24 h. A dose of mutagenizing drug is used that causes 80-95% cell death. After drug treatment, the surviving cells are allowed 5-7 days to recover before transfer with trypsin to undergo selection or for direct observation for the appearance of flat colonies.

(2) Extraction of high molecular weight DNA is carried out by the method of Gross-Bellard et al. The DNA is cut with restriction enzymes and analyzed by Southern blot using a probe for Kirsten DNA.

(3) Selection of revertants is carried out in suspension culture in 0.9% methyl cellulose using 5-fluorouracil to kill dividing cells. The surviving cells are recovered with the aid of cellulase enzyme, plated at 200 cells/dish and observed for the appearance of flat colonies. Such colonies are isolated by means of cloning cylinders with silicon grease applied to one end. The cloning cylinder adheres to the plastic surface of the dish so that localized trypsin can be used to dislodge the cells within the confinement of the cylinder. Another method of selection uses the increased sensitivity of the transformed cells to ouabain. Incubation in the presence of ouabain thus increases the ratio of revertant over transformed cells.

Major Findings:

The double transformed Kirsten NIH 3T3 cell contains two copies of the Kirsten genome. It is known that mutagenesis of cells transformed by RNA-tumor viruses results in a high frequency of revertants from which the transforming virus can no longer be rescued. Apparently, sequences in the viral genome serve as a preferential target for the mutagen drug. As our goal was to isolate revertants affected at a cellular rather than at a viral function, such revertants would be an undesirable background. To overcome this problem, we have used a cell line of NIH 3T3 cells that have been infected two consecutive times with Kirsten virus. Analysis of the DNA of this cell line indeed showed that it contains two copies of the Kirsten viral genome. Theoretically, the chance to inactivate the virus in a cell with two viral copies is multiplication of the chances to inactivate the virus in a cell with only one viral copy.

NIH 3T3 cells transformed with Kirsten and Harvey DNA are more sensitive to ouabain than untransformed cells. It was recently reported that the increased aerobic glycolysis in Erlich ascitis tumor cells is due to inefficient action of the cell membrane Na^+,K^+ ATPase. To test whether the Na^+,K^+ ATPase of Kirsten and Harvey transformed NIH 3T3 cells is also inefficient, Kirsten transformed cells and untransformed NIH 3T3 cells were incubated with various concentrations of ouabain, a specific inhibitor of the Na^+,K^+ ATPase. It was found that the transformed cells are much more sensitive to ouabain than the untransformed NIH 3T3 cells. At sufficiently high doses (1 mM), ouabain causes rounding and eventual lysis of the transformed cell with only slight effects on the NIH 3T3 cells. The killing of transformed cells by ouabain is probably due to K^+ starvation since decreasing the K^+ concentration in the medium greatly potentiates the effect of ouabain. To test whether the sensitivity to ouabain is a general property of NIH 3T3 cells transformed by Harvey and Kirsten, NIH 3T3 cell cultures transfected by Harvey or Kirsten DNA were cultured in the presence of ouabain. In these experiments, ouabain caused lysis of the transformed foci while leaving the layer of untransformed cells intact. Since each focus represents a different cell line, it can be concluded that the sensitivity to ouabain is related to transformation by Harvey and Kirsten viruses and is not a property of a particular cell line. Using cloning efficiency as a quantitative parameter, it was determined that the Kirsten transformed cells are about one hundred times more sensitive to ouabain than NIH 3T3 cells.

Significance to Biomedical Research and the Program of the Institute:

The virus-induced transformation of normal target cells is believed to be mediated by transforming proteins coded for by the viral genome. The evidence for this notion comes from genetic experiments with temperature-sensitive mutants for transformation. In the case of Kirsten transformed cells, it was shown that a temperature-sensitive mutant for transformation produces p21 protein which is temperature-sensitive in its ability to bind guanine nucleotides. Little information is available, however, on the relevant cellular targets for the transforming proteins. The main problem is that too many changes occur upon transformation and it is difficult to establish a cause and effect relationship between these changes and transformation. Isolation of cellular mutants that are modified at a cellular target for the transforming protein could be very useful for analysis of the molecular events which lead to transformation. These studies recently gained impetus by the discovery of a cascade of protein kinases that appear to be a target for several src gene products of various transforming viruses. Elucidation of the transformation pathway may be very important for future design of drugs that specifically act on transformed cells and thus lead to more rational therapy of cancer.

Proposed Course:

Efforts will be made to characterize the isolated mutants and unravel the modified reactions.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05183-01 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of the Harvey Sarcoma Virus src Protein with Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. E. Furth	Staff Fellow*	LTVG NCI
OTHERS:	E. M. Scolnick	Chief	LTVG NCI

*Special Fellow of Leukemia Society of America, 10/1/80-2/1/81

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Harvey and Kirsten murine sarcoma viruses (HaSV and KiSV) carry related src genes, each of which is a representative of a small family of conserved cellular sarc genes. The src gene of each virus encodes a protein of approximately 21,000 daltons (p21) which can induce malignant transformation in infected cells. The HaSV and KiSV src proteins are related immunologically, both to each other and to the endogenous p21 species expressed in a variety of vertebrate cells. In order to facilitate the identification and functional characterization of different p21 species, we are preparing potent and specific monoclonal antibody reagents directed against these proteins. We find that some monoclonal antibodies to the HaSV src protein bind only to p21 species encoded by the most closely related genes, while others bind to more divergent species as well. These reagents should therefore permit purification of specific forms of p21. We are particularly interested in expression of p21 in hematopoietic cells, and hope to use the antibodies to study the action of p21 in transforming erythroid cells. We also wish to study the potential role of particular p21 species in the control of normal hematopoietic growth and cell differentiation.

Project Description

Objectives:

The transforming genes of a number of retroviruses are derived from normal cellular genes which have been conserved throughout vertebrate evolution. The precise mechanism by which the expression of such "onc" genes under viral control leads to the loss of cellular growth control remains unknown, as does the function of these genes in normal cell growth and differentiation. A major goal in the understanding of viral carcinogenesis is to characterize the protein products of onc genes with respect both to regulation of expression and to function in cellular metabolism. Important tools in such studies have been antisera raised in animals bearing viral-induced tumors. Such sera contain antibodies specific to the viral-encoded onc protein and, in some cases, to the equivalent cell-encoded protein(s). Although extremely useful, these antisera are often limited both in potency and in specificity for the proteins of interest. We therefore have sought to produce improved antibody reagents of extremely high titer and specificity by employing the "hybridoma" technique of Kohler and Milstein to isolate cell clones that will secrete a single antibody species directed against a particular onc gene product and will grow indefinitely.

We have focused on the src gene products of the Harvey and Kirsten strains of murine sarcoma virus (HaSV and KiSV) and on the products of the related cellular sarc genes. The two viral src genes are representatives of a small family of divergent vertebrate genes. Each known member of the gene family encodes a polypeptide of approximately 21,000 daltons (p21) which is associated with a guanine nucleotide binding activity. The first goal of this study is to obtain monoclonal antibody reagents directed against the p21 encoded by HaSV and to determine whether such reagents can be used to distinguish among the genetically divergent viral and cellular-encoded species of p21 proteins. The antibodies would also facilitate the purification and functional characterization of the various p21 species. Because HaSV and KiSV, in addition to inducing sarcomas, can induce erythroleukemia in mice and erythroid transformation in bone marrow cells in vitro, and because at least one murine hematopoietic cell line (416B) expresses high levels of an endogenous p21 species, we are particularly interested in studying p21 expression during hematopoietic cell differentiation. A long range goal of these studies is to test the hypothesis that particular sarc genes may help to control proliferation and differentiation in specific cell lineages and to determine whether sarc gene expression influences the potential of particular cell types to undergo malignant transformation.

Methods Employed:

(1) Cell fusion of splenic lymphocytes of immune rats with rat myeloma cells, and cloning of hybrid antibody-producing cells.

(2) Immunofluorescence microscopy.

(3) Immunoprecipitation and SDS-polyacrylamide gel electrophoresis for analysis of radioactively labeled antigens in cell extracts.

(4) Immunoaffinity chromatography.

(5) Agarose gel electrophoresis of RNA, blot transfer to activated paper, and hybridization of ^{32}P-labeled cloned DNA probes.

Major Findings:

(1) Monoclonal antibodies to the HaSV src gene product have been obtained by fusion of splenic lymphocytes from immune tumor-bearing rats to rat myeloma cells and cloning of active hybrid cells.

(2) Some monoclonal antibodies bind forms of p21 most closely related to the src gene product of HaSV, but do not bind those related to the src product of KiSV. Other monoclonal antibodies appear to bind all of the known genetically distinct forms of p21 present in mouse and rat cells.

(3) A murine hematopoietic cell line (416B) which expresses high levels of an endogenous p21 contains elevated levels of messenger RNA homologous to the src gene of KiMSV, but not to that of HaSV. In addition, the p21 of 416B cells is antigenically related to that encoded by KiSV, as shown by cross-reation with a monoclonal antibody.

Significance to Biomedical Research and the Program of the Institute:

It is not yet known how the oncogenes (onc genes) carried by some retroviruses transform cells to malignancy, how the equivalent cellular genes act in normal cell growth, and whether changes in expression of such cellular genes might lead to neoplastic transformation. The production of potent and specific monoclonal antibody reagents directed against particular onc gene products would facilitate studies of the expession and function of these proteins. Because a number of onc genes appear to be evolutionarily conserved, it is likely that some monoclonal antibodies directed against the transformation-inducing proteins identified in animal model systems will also bind to equivalent proteins in human cells. The reagents may therefore be useful in assessing the role of oncogene expression in human cancer.

Proposed Course:

(1) Isolate monoclonal antibodies to HaSV-encoded p21.

(2) Identify cross-reations of such antibodies with divergent forms of p21.

(3) Isolate additional monoclonal antibodies, as needed, to KiSV p21 and to the p21 species expressed in the murine hematopoietic cell line 416B.

(4) Identify cells expressing p21 in normal hematopoietic populations and after infection with HaSV or KiSV.

(5) Distinguish among endogenous species of p21 expressed in hematopoietic cells (including the 416B cell line).

(6) Purify various species of p21 by immunoaffinity chromatography in order to facilitate functional studies.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05184-01 LTVG

PERIOD COVERED
December 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Molecular Basis of Disease Induced by the FVA Strain of the Spleen Focus-Forming Virus.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Kaminchik	Visiting Fellow	LTVG	NCI
OTHERS:	E. M. Scolnick	Chief	LTVG	NCI
	D. L. Linemeyer	Staff Fellow	LTVG	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Based upon their pathogenicity, two strains of Friend erythroleukemia virus were isolated in the past: the FVA strain which causes anemia in susceptible mice and the FVP strain which causes polycythemia. Both FVA and FVP stocks contain two distinct components: a replication defective virus, the spleen focus-forming virus (SFFV), and an ecotropic murine leukemia virus which serves as a helper (F-MuLV). The defective SFFV was shown to induce an erythroproliferative disease in adult mice, whereas the F-MuLV helper was capable of inducing leukemia only in newborn mice. Studies were initiated to determine the genetic and molecular differences between the FVA and FVP strains of SFFV which may account for the differences in their biological properties. Presently, attempts are being made to clone a biologically active form of FVA DNA in E. coli using a plasmid as a vector.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The purpose of this project is to identify the gene(s) responsible for the induction of anemia by the FVA strain of Friend spleen focus-forming virus, and examine the molecular differences between this gene and that responsible for the induction of polycythemia in FVP strains.

Methods Employed:

1. Cloning of FVA DNA will be done by *in vitro* recombination with *E. coli* plasmid pBR322 and transformation of *E. coli* with the recombinant plasmid.

2. Cloned FVP DNA and subgenomic clones of known genetic capacity will be used as probes for establishing a genetic map of the cloned FVA DNA.

3. DNA transfections into fibroblasts and induction of disease in mice by injecting virus rescued from transfected fibroblasts will be used in studying the genetic properties of the cloned FVA DNA.

Significance to Biomedical Research and the Program of the Institute:

A better knowledge of gene organization and genetic capacity of the FVA DNA will be helpful in understanding its biological properties and the difference in pathogenicity between the FVA and FVP strains of the spleen focus-forming virus. *In vitro* recombination studies between the genomes of the two viruses will allow us to determine the role of different genes in the induction of either anemia or polycythemia in mice.

Proposed Course:

1. Unintegrated FVA DNA was isolated from freshly infected cells. That DNA will be molecularly cloned in *E. coli* using the plasmid pBR322.

2. A restriction enzyme map of the cloned DNA will be established and used for further cloning of subgenomic DNA fragments.

3. The genetic map will be developed using cloned FVP DNA fragments of known genetic capacity.

4. The biological activity of cloned FVA DNA will be determined by DNA transfections into fibroblasts and by injecting rescued virus from those cells into mice.

5. _In vitro_ recombination of various portions of the FVA and FVP DNAs will be done. The biological properties and pathogenicity of the newly derived viruses will be studied.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05185-01 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mechanisms of Thymic Leukemia Induction in Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: L. F. Hu	Visiting Fellow	LTVG NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Biochemistry Section .

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

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1.0	1.0	0

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SUMMARY OF WORK (200 words or less - underline keywords)

Mo-MuLV-induced rat thymomas are oligoclonal tumors. A 3' LTR-cell DNA junction fragment is repeated size wise in 60% of the tumors. This fragment has now been cloned and will be examined as a potential transforming gene induced by the introduced viral promoter.

Project Description

Objectives:

The overall objectives of this project are to define and analyze biologically important DNA sequences in Mo-MuLV-induced rat thymomas.

Major Findings:

High molecular weight DNA from Mo-MuLV induced rat thymomas was digested with EcoRI, a restriction endonuclease that does not cleave within the Mo-MuLV genome. Southern blotting and hybridization with a ^{32}P labeled nick translated Mo-MuLV probe showed that the tumors were oligoclonal. The number of hybridizing DNA bands was invariably large and ranged from 5-15. These data were confirmed using two more restriction endonucleases (PstI and XhoI) that cleave only once within the Mo-MuLV genome. For this last set of experiments the probe used was a BamHI-SalI 2.7 kb fragment subcloned from Mo-MuLV DNA in pBR322. This probe hybridizes only to the 3' half of the Mo-MuLV genome and therefore detects only the 3' end function fragments.

Our working hypothesis is that tumors induced by nontransforming type C retroviruses are due to insertion of the viral promoter next to a potentially transforming cellular gene, promoting its active transcription. In the case of Mo-MuLV induced rat thymomas, due to the large number of virus integration sites in each tumor, it is very difficult to identify the integration site responsible for oncogenesis. One possibility would be that if the same integration site appears in more than one tumor, it could be significant for oncogenesis. From the analysis of the tumor DNA described in the first paragraph, we were unable to identify common bands between tumors. However, this could be due to the fact that many of the integrated proviruses may be defective carrying extensive deletions as it has been shown in the case of ALV induced tumors. In order to avoid this problem, we repeated the Southern blotting analysis of the tumor DNA using the restriction endonuclease SacI that cleaves within the Mo-MuLV large terminal repeat (LTR) and either a U_5 probe or a probe that would detect only internal viral sequences. This approach allowed us to identify a .9 kilobases long 3' LTR-cell junction fragment, that size wise was repeated in 60% of the tumor DNAs. This junction fragment has now been cloned in a lambda gtWES•λB•SacI vector. We are currently analyzing this clone and its significance for oncogenesis.

Significance to Biomedical Research and the Program of the Institute:

This work aims to the understanding of the mechanism by which Mo-MuLV-induced thymomas arise in rats. However, understanding the events involved in this system could provide the conceptual framework for the understanding of nonviral-induced tumors in other mammalian species, including humans. In addition, cloning the cellular genes involved in this process could provide valuable tools to be used in research on human malignancies.

Proposed Course:

We are planning to examine the significance of the clone available in the generation of Mo-MuLV-induced rat thymomas, by testing the physical linkage of this gene to Mo-MuLV in the DNA level, and by evaluating its expression in the tumor RNA. In addition, its ability to cause transformation by transfection of NIH 3T3 cells, its sequence homology to known transforming genes will be evaluated. After this is documented, we will examine its distribution and genetic organization between species and its expression in various tissues and differentiating cells of the same species. The expression of this sequence in a variety of viral and nonviral tumors from various species, including humans, will also be evaluated.

Finally, the clone will be sequenced. The most important reason for sequencing is to identify any open reading frame(s) present, and to ask questions regarding the protein that might be encoded by this sequence.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05186-01 LTVG

PERIOD COVERED
November 5, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Expression and Regulation of Avian RNA Tumor Viruses Transforming Genes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. P. Samuel	Visiting Fellow	LTVG	NCI
OTHER:	T. S. Papas	Head, Carcinogenesis Regulation Section	LTVG	NCI
	J. A. Lautenberger	Expert	LTVG	NCI
	K. Rushlow	Guest Worker	LTVG	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Specific initiation of transcription of avian myeloblastosis virus by DNA polymerase II was obtained in a cell-free system using clonal AMV DNA as a template. The site of initiation is located in the U_3 region of the long terminal repeat (LTR). This indicates that the information necessary for AMV transcription lies within the viral genome.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The scope of this investigation is to delineate the relationship between virus gene expression and conversion of cells from normal to malignant state and to study the molecular anatomy of known tumor viruses and describe the mechanism by which subviral structures act in concert with cellular factors to regulate oncogenesis. The technique of molecular cloning, DNA sequence analysis, and site-specific mutagenesis will be used to implicate specific nucleotides in the transformation process.

Methods Employed:

A. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by *in vitro* packaging. Isolation of phage of libraries containing virus-related sequences by hybridization of cDNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.

B. Construction of Recombinant Plasmids. Double-stranded cDNA of AMV genome was prepared. It was converted to perfect duplex molecules with blunt ends by digestion with single-strand nuclease S_1. It was then incubated with *E. coli*. DNA polymerase I. The product of the S_1-DNA polymerase reaction was ligated to Bam HI linkers with T4 DNA ligase. pBR322 was digested with Bam HI, treated with alkaline phosphotase. It was then ligated to double-stranded cDNA linked to Bam HI linkers.

C. Transformation and Identification of Recombinant Clones. Construction of chimeric plasmids and the transformation of *E. coli* X1776 by these plasmids was performed in a p2 physical containment laboratory. X1776 was transformed by a transfection procedure. Transformed colonies containing AMV sequences were identified by colony hybridzation. The colonies were screened with ^{32}P-labeled AMV cDNA. Strongly hybridized colonies were selected and replated; single colonies were picked and grown.

Major Findings:

A. Screening of human library for presence of MC29 specific sequences. Library of partial EcoRI digests was obtained from T. Maniatis. Using MC29 specific nick translated probes we have screened this library. We have identified two potential clones containing homologous MC29 specific sequences. We are presently characterizing these clones by restriction enzyme analysis and heteroduplex mapping.

BEST DOCUMENT AVAILABLE

B. Specific initiation of transcription of avian myeloblastosis virus by DNA polymerase II was obtained in a cell-free system using clonal AMV DNA as a template. The site of initiation is located in the U_3 region of the long terminal repeat (LTR). This indicates that the information necessary for AMV transcription lies within the viral genome.

Significance to Biomedical Research and the Program of the Institute:

It is clear that the formation, the maintenance and the expression of provirus are the central features of the life cycle of RNA tumor viruses. Many questions related to these aspects are unsettled. To elucidate the process of oncogenesis induced by these viruses it is important to understand the structural organization of the transforming genes within the host chromosome and the process by which these genes are expressed and regulated.

Proposed Course:

1. To characterize the human MC29 clone and compare its structure to the viral clone. To test the biological activity of these clones.

2. To devise an *in vitro* transcription translation system in an effort to synthesize the transforming protein of both AMV and MC29.

Publications:

None

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05187-01 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Recombination Mechanisms between Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G. Strauss	Guest Worker	LTVG NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two biologically important regions of the genome of the type C retroviruses, e.g., the U_3 region and a region that maps between env and src, are investigated for their role in transformation by transforming nontransforming retroviruses. New recombinant avian retroviruses are being isolated and their structure, replication rate, and oncogenic activity will be determined.

BEST DOCUMENT AVAILABLE

Project Description

Objectives:

The overall objectives of this project are to define biologically relevant sequences in the genome of type C retroviruses and analyze their role in the control of replication transformation and oncogenesis.

1. Determine the role of the U_3 region in transformation by nondefective transforming type C retroviruses and oncogenesis by their nontransforming counterparts.

2. Determine the role of a region that maps between *env* and *src* in transformation by nondefective transforming viruses and in oncogenesis induced by their nontransforming derivatives.

Major Findings:

1. A mixture of $\underline{src}^{+}U_3^{end}$ and $\underline{src}^{+}U_3^{exog}$ viruses generated by recombination between RAV-0 and PzRSV-B (MRE4) was used to infect QT_6 cells. The nonintegrated circular viral DNA from acutely infected cells was cloned in λgtWES·λB SalI lambda vector. One hundred (100) independent $\underline{src}^{+}$ viral clones have been isolated. We are now involved in the process of screening these clones in order to identify $\underline{src}^{+}U_3^{end}$ recombinants.

2. A genetic region that maps between the *env* and *src* genes of the nondefective transforming avian retroviruses has been shown to play a role in transformation. The same region is probably involved in leukemogenesis by replication competent nontransforming retroviruses. We are now in the process of studying the function of this region.

Significance to Biomedical Research and the Program of the Institute:

The aim of this work is to provide an understanding of the mechanism of transformation by type C retroviruses. This understanding will provide a conceptual framework important for the understanding of nonviral transformation.

Proposed Course:

We will first screen the recombinant clones that we have so far generated in order to identify $\underline{src}^{+}U_3^{end}$ recombinants. Any such clones will be tested for their ability to induce transformation when transfected in a variety of cells. If these clones cannot induce transformation, we will investigate the possibility that they may become transformation competent upon recombination with $\underline{src}^{-}U_3^{exog}$ leukosis viruses. In addition, we will generate $\underline{src}^{-}U_3^{end}$ viruses by passaging the $\underline{src}^{+}U_3^{end}$ clones in tissue culture. These viruses will be tested for their ability to induce disease in chickens.

BEST DOCUMENT AVAILABLE

Regarding the role of the region that maps between env and src in transformation, we plan to analyze cells infected with two viral recombinants that differ in this region while they are otherwise identical. As a first step, we plan to examine the src protein and RNA expression in these cells. Further investigation will depend on the data we will obtain.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05188-01 LTVG

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Viral Leukemogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Zeller	Guest Worker	LTVG NCI

COOPERATING UNITS (if any)

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The activation of endogenous transforming genes during normal cellular differentiation, as well as viral and nonviral malignancies, was investigated. Some human lymphoid malignancies express sequences homologous to the onc gene of MC29 avian leukemia virus.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The objective of this project is to study the role that specific endogenous transforming genes play in viral and nonviral induced malignancies as well as in normal cellular differentiation. The specific goals are:

1. To detect activated endogenous transforming genes in Moloney leukemia virus-induced rat thymomas.

2. To determine if endogenous genes known to be constitutively expressed in avian leukosis virus-induced B cell lymphoid malignancies are also expressed during normal cellular differentiation.

3. To establish if endogenous transforming genes in the human are also expressed and play a role in the development of malignancies.

Major Findings:

1. High molecular weight DNA from Mo-MuLV-induced rat thymomas (see report by P. N. Tsichlis and L. F. Hu) was transfected in NIH 3T3 cells, in order to examine its ability to transform these cells in culture. Foci of transformed cells were observed and scored two weeks later. The efficiency of transformation was 0.1-2 foci per μg of DNA. Individual clones of transformed cells were frozen in liquid nitrogen. We plan to reexamine these clones for the mechanism by which they transformed as soon as the necessary probes are available.

2. Ninety percent of the tumors induced by avian leukosis viruses (ALV) are B cell lymphomas and in the majority of these tumors examined, the ALV provirus promotes the transcription of endogenous MC29 sequences. These findings suggest a possible connection between B lymphoid cells and expression of the endogenous MC29 gene. Our preliminary findings indicate that the endogenous MC29 RNA is expressed in the bursa of fabricius of chicken embryos and not in other tissues, suggesting that the MC29 gene may be involved in the normal differentiation of B lymphoid cells.

3. The process of transformation induced by ALV may occur similarly in humans, i.e., in specific types of tumors, specific endogenous transforming genes become activated. We have examined a variety of human lymphoid tumors for the expression of endogenous MC29 sequences. Many tumors, including diffuse histocytic lymphomas, a Burkitts lymphoma, and some poorly differentiated B cell lymphomas, have been found to express MC29.

Significance to Biomedical Research and the Program of the Institute:

The transforming genes of retroviruses have a normal cellular counterpart which is highly conserved throughout evolution. Most transforming retroviruses cause disease by introducing as exogenous copy of the transforming gene into the host. However, ALV transforms cells by activating endogenous transforming genes. The research presented in this report seeks to identify the process of activation of endogenous transforming genes in human malignancies as well as in other animals. The work we describe will not only identify this process, but also will provide information on the target cells' specificity and the mechanism by which the genes are activated. We hope to be able to demonstrate that the process of oncogenesis induced by retroviruses in animal models can be directly related to the development of malignancies in humans.

Proposed Course:

We will contine our efforts to identify the transforming gene(s) involved in the development of Mo-MuLV-induced rat thymomas. Any probes generated through these efforts will be used to examine the clones of transformed NIH 3T3 cells obtained by transfection of tumor high molecular weight DNA.

We will also continue the screening of the developing bursa and other chicken embryo tissues for the expression of MC29 during various stages of development.

The expression of MC29 in human tissues will be further investigated. Future plans will depend upon the results of the work now in progress.

Publications:

None

CONTRACT NARRATIVE
CARCINOGENESIS INTRAMURAL PROGRAM
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1981

Laboratory of Tumor Virus Genetics

MELOY LABORATORIES (NO1-CP-01013)

Title: Immunological and Biochemical Studies of Mammalian Viral Oncology

Contractor's Project Director: Dr. Kenneth Blackman

Project Officers (NCI): Dr. Edward M. Scolnick and Dr. Robert J. Goldberg

Objectives: The purpose of this contract is to provide resource support and technical services to the Laboratory of Tumor Virus Genetics, Carcinogenesis Intramural Program, Division of Cancer Cause and Prevention, National Cancer Institute. The areas of support include cell culture, virology, biochemistry, immunology and recombinant DNA technology.

Methods Employed: Various mammalian cell lines and primary cultures are propagated using standard and, where indicated, specialized tissue culture procedures including cell cloning and screening for mycoplasma and other extraneous contaminating agents. Virus stocks are grown, partially purified and titrated using the XC, S^+L^- and other infectivity assays. Assays for viral reverse transcriptase activity are performed on the supernatant fluids of infected and control cell cultures using the exogenous template $rA\text{-}dT_{12-18}$ and radioactive TTP. Both viral and cellular nucleic acids and proteins are isolated and purified utilizing various biochemical techniques including differential centrifugation, affinity, ion-exchange and gel chromotography, immunoprecipitation and gel electrophoresis. The contractor has maintained the capability to perform multiple radioimmunoassays on a weekly basis. Hybridoma cell lines are prepared for production of monoclonal antibodies directed against various murine RNA tumor virus determinants. The recombinant DNA support facility is responsible for the growth, harvest, purification and characterization of bacterial-containing recombinant DNAs using biological and molecular techniques specified by NCI investigators.

Major Findings: More than one dozen cell lines and experimental cell cultures have been maintained on a weekly basis. These cultures have been used for the growth and assay of RNA tumor viruses, transfection assays, generation of hybridoma cells lines and biological substrates for purification of virus and cellular nucleic acids and proteins. Assays for RNA-directed DNA polymerase activity have usually included the daily processing of 20 to 50 samples including appropriate controls. Virus and virus-related proteins have been purified for use as reagents for radioimmunoassays and for the preparation of specific antisera. Efforts have been expended to develop hybridomas for production of monoclonal antibodies to Friend murine leukemia virus and Harvey sarcoma virus structural and transforming proteins. A specific rapid screening assay was employed for detection of monoclonal antibodies in the supernatant fluids of hybridoma cultures utilizing iodinated protein A and autoradiography for detection of specific antigen-antibody complexes. During the past year, several MCF envelope protein and gag-specific hybridomas have been isolated which are currently being evaluated. A major emphasis of the LTVG research program has been on the molecular cloning of RNA tumor viruses using plasmid and bacteriophage vectors. The recombinant DNA support facility at Meloy Laboratories has prepared a variety of reagents for these studies including purified plasmid DNAs isolated by the Birnboin procedure, lambda DNA, lambda vector arms, protein A packaging extracts and specific DNA sequences isolated by gel electrophoresis. All plasmids were digested with the appropriate restriction endonucleases and analyzed by gel electrophoresis. The contractor has also acquired the capability to transform E. coli with plasmid DNAs and perform filter screening assays of resultant colonies using appropriate probes.

Significance to Biomedical Research and the Program of the Institute: Tumor viruses are recognized as valuable reagents for defining the molecular pathways leading to malignant transformation. The isolation of viral transforming proteins and the identification of the genetic sequences that code for these proteins have provided reproducible means by which the molecular mechanisms of cancer can be studied. It is now clear that retrovirus transforming genes are derived by recombination with normal cellular genes which are highly conserved in all vertebrate species including man. These studies are the mainstay of the research program of the Laboratory of Tumor Virus Genetics and this contract supplies indispensable resource support and technical services not presently available on the NIH reservation to this critically important area of cancer research.

Proposed Course: The present contract is expected to continue through November 19, 1984. Primary emphasis will be placed on those resource support activities related to LTVG research using DNA recombinant and hybridoma technology. No major changes in the workscope of the contract are presently planned.

Date Contract Initiated: May 1980

Current Annual Level: $720,000

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SUMMARY REPORT

LABORATORY OF VIRAL CARCINOGENESIS

October 1, 1980 to September 30, 1981

The Laboratory of Viral Carcinogenesis (LVC) is charged with the planning, development, implementation, and coordination of the Institute's research programs in carcinogenesis with special emphasis on delineating the role of oncogenic viruses, virus-related genetic sequences in human cancer, and the general mechanisms of cellular gene control. Research efforts are conducted on virus-host relationships in virus-induced cancers with emphasis on the detection and characterization of oncogenic viruses, their integrating genetic tracts, and their mode of transmission in animals and man. The host immune mechanism is studied in relationship to its genetic control, especially in virus-related cancers. Investigations are conducted on the molecular processes in carcinogenesis and cocarcinogenesis. Viruses are used as the most precise tools for the analysis of gene control and malignant transformation. Activities of the Laboratory are conducted by in-house research and collaborative agreements with other research organizations.

This year included a significant transition for the LVC. In October all of its sections, formerly scattered both within and outside of the NIH Bethesda campus, were moved and consolidated into a single location, Building 560, at the Frederick Cancer Research Center (FCRC), Frederick, MD. After the move several laboratories were housed in temporary quarters at the FCRC while necessary architectural and utility modifications were made. Final accomodations were completed approximately by July 1, 1981. These facilities at the FCRC do not yet include space assigned to the LVC for the housing, maintenance and testing of experimental animals. Animal facilities are scheduled to be phased in sometime during FY 83. In the meantime experimental animals continue to be housed at various off-site locations under contract arrangements. In spite of these disruptions and personnel shifts natural with a move of this magnitude, the research momentum was maintained.

Results described in this report strongly support the concept that malignant transformation in humans is most probably a multistage, multifactorial progression of internal and external events which follow well-established metabolic pathways governed by molecular, enzymatic and genetic controls as well as mechanisms mediated by definable, biologically active molecules. It appears further that the capacity to start, promote, and accelerate such progression depends strongly on the mosaic genetic constitution intrinsic to a given host; some genetic information naturally conferring protection while another increases risk. Among these endogenous host factors are the presence, absence, control, and gene-dose of certain DNA sequences, integrated in the host's chromosomes, which are related to known oncogenic mammalian and primate viruses and other, possibly nonviral (cellular), information that can express intermediate molecules, hormone-like substances which can induce phenotypic changes related to malignancy in cells. Some exogenous factors may include chemical carcinogens, tumor promoters, and oncogenic viruses. This Laboratory seeks to elucidate the basic mechanisms of cancer induction and progression in order to harness them in ways useful in the prevention or modification of a cell's progression towards cancer. The most significant findings obtained during this year can be clustered as follows: (A) the identity, origin, transmission, structure, function and virulence of proviral and cellular DNA sequences in mammals, primates, and possibly humans; (B) the identity, nature and function of hormone-like control and signal factors that mediate the genetic and phenotypic expression of malignancy in mammalian cells; (C) the identification and characterization of similar factors that specifically interfere with the host's defensive physiological responses to cancer; (D) the metabolic pathways and intermediate products involved in endogenous transformation, tumor promotion and cocarcinogenesis; and (E) the results of epidemiological studies on human cancers with associated viral etiologies.

A. Identity, Origin, Transmission, Structure, Function and Virulence of Proviral and Cellular DNA Sequences in Mammals, Primates, and Possibly Humans.

The discovery and evolutionary tracing of numerous retroviruses by this Laboratory has served to better classify these viruses and led naturally to the isolation and classification of a variety of viruses from the primates most closely related to man. This year, for example, Colobus monkey DNA was determined to contain at least three distinct sets of type C virus-related sequences. Other primates such as the baboon and rhesus monkeys contain at least two distinct sets, one related to the M7 baboon virus and another to the CPC-1 colobus monkey retrovirus. Quite surprisingly, chimpanzee DNA was found to contain only a single family of sequences related to both viruses. These chimpanzee sequences, obtained from the closest living relative of man, could be expected to provide the best DNA hybridization probes for the detection of related endogenous viral sequences in the DNA of man. Such probes easily detected related sequences in cellular DNA from gorilla, baboon, rhesus, colobus monkeys and even mice but did not detect them in man, gibbon or orangutan. Clearly the chimpanzee has either never had these sequences or has now lost them. It is highly improbable that such sequences were ever present in the human-chimpanzee ancestor or that they have diverged rapidly enough to escape present detection. This demonstration leaves us with the strong probability that unknown human viral sequences might be detected, not by using phylogenetically predictable viral probes but rather by probes from viruses from more distantly related species. If this is the case, then there is no obvious predictive preference for using one virus rather than another.

The major internal protein (p30) of MAC-1, an endogenous type C virus of *Macaca arctoides* and the p30 of CPC-1, an endogenous type C virus of *Colobus polykomos*, were purified and subjected to primary structure analysis. Despite the distant evolutionary relationship (approximately 20 million years) between these two species of Old World monkeys, the amino acid compositions of the viral p30s were very similar and their COOH-terminal sequences (5 residues) were found to be identical. Moreover, the NH_2-terminal sequences (up to 36 residues) differed only in three positions. Both the NH_2- and COOH- terminal sequences showed extensive homology to respective sequences of p30s of known type C viruses from other mammalian species. On the basis of these p30 sequence relationships, MAC-1 and CPC-1 together with MMC-1, an endogenous virus of *Macaca mulatta*, can be classified into a new subgroup which is related to the avian reticuloendotheliosis virus and its relatives, transforming viruses of birds. The 5' terminal nucleotide sequence regions of CPC-1 and MAC-1 show a 76% nucleotide correspondence and are 132 and 127 nucleotides long, respectively. Previous strong stop analysis of other type C viruses have defined two subgroups, Rauscher murine leukemia virus/gibbon ape leukemia virus and baboon endogenous virus/feline rhabodomyosarcoma virus (RD-114). Based on our sequence analysis of their 5' terminal sequences, CPC-1/MAC-1 form a subgroup. Comparison of CPC-1 and MAC-1 to the sequence of avian spleen necrosis virus (SNV), a member of the reticuloendotheliosis virus group, adds SNV to this subgroup of mammalian type C viruses. CPC-1/MAC-1 and SNV contain conserved regulatory signals in similar positions in their 5' terminal RNA sequences analogous to those observed in other mammalian type C retroviruses. These sequences include the canonical AAUAAA sequence, a palindrome, a putative ribosome binding site, and an integration site. Some of these highly conserved subsequences are common to 3' and 5' terminal noncoding sequences of nonviral eukaryotic messenger RNAs.

Trophoblasts in primate placentas were studied for gene expression related to primate retrovirus. Antigen expression for internal structural protein (p26) from Macaca arctoides type C retrovirus (MAC-1) was detected in 16 out of 16 placental specimens (2 to 218 ng/mg protein) but not in 10 other different fetal organs from each of 8 selected animals. This showed expression of proviral genes in a non-malignant tissue perhaps serving a "normal" function yet, underscoring its presence in the cellular genome.

To gain insight into the genomic organization, gene function, and gene expression of oncogenic viruses in mammalian cells, DNA copies of the RNA tumor viruses were obtained using recombinant DNA technology. Cloned DNA molecules facilitate the study of viral and cellular genes at the cistron level and permit the manipulation of the genomes at strategic regions involved in tumorigenesis and leukemogenesis. For example, the frequency and organization of the retrovirus CPC-1 within the normal colobus monkey DNA was defined. The complete CPC-1 virus was cloned and mapped first as three non-overlapping EcoRI restriction fragments. This was used to detect and compare related sequences integrated in the monkey cellular DNA. Approximately 100 copies of CPC-1-related DNA units were found and most of these had the same restriction sites as the cloned viral DNA. Eighteen of 19 restriction sites were found to be highly conserved in the cellular sequences and the integrated proviral sequences exist essentially intact. Concomitantly, transfection analyses can show exactly which genomic regions are required for biological activity and the protein products can be predicted from the DNA structures. This demonstrated that specific viral promoter regions at the ends of proviral DNA are critical not only for virus expression, but can also effect the promotion of cellular genes. These reiterated proviral specific terminal sequences are generally referred to as "long terminal repeats" (LTR). This effect permits construction of plasmids containing desirable nontransforming genes programmed for a high degree of expression of selected sequences. Construction of specific marker LTR molecules then serves to readily identify immunologically those rare cells transfected with a specific sequence of interest. Furthermore, retroviruses can be used as true vectors for genes other than those that lead to oncogenesis (e.g., a transmissible recombinant retrovirus containing the thymidine kinase gene of Herpes simplex virus type I).

Subgenomic fragments of murine sarcoma virus (MSV) proviral DNA cloned in pBR322 were used to transfect and transform mouse cells. Upon superinfection with murine leukemia virus (MuLV), infectious MSV could be recovered if the MSV DNA transfecting fragment consisted both of LTR and the MSV specific v-mos (mos from virus, formerly src) nucleotide sequences. The virus specific RNAs appear to contain not only MSV sequences, but also cloning vector sequences indicating that prokaryotic and oncoviral sequences can be functionally linked. Infectivity of inefficiently transforming MSV proviral DNA segments can then be enhanced several hundred-fold when viral LTRs were added. The normal mouse cell contains a single DNA sequence (c-mos) which is essentially identical to v-mos. By itself, the cloned c-mos did not transform cells in transfection, but when LTR's were linked to the c-mos, effective transformation was observed. The viral LTR, presumed to contain promoter type sequences, was absolutely needed for biological activity. Thus, a viral putative promoter could activate normal, inactive cellular genes.

The LTR from CPC-1 was partially sequenced to determine the nature of its presumptive promoter region. Two canonical "TATA" boxes were observed, 29 and 60 nucleotides upstream from the transcription initiation site (cap site, defined from the sequence of strong stop DNA). Two 9-nucleotide sequences (CCAATCATA) approximately 55 nucleotides upstream from each "TATA" box were also identified. Whereas the later sequences may be involved in the binding of RNA polymerase, hence effecting the efficiency of transcription, the former most likely determine the cap site for transcription. The duplicitic nature of such promoter regions suggested that its transcription efficiency might be quite high. This was substantiated when productively infected A549 cells revealed the presence of an extraordinarily high number of viral transcripts (5-10,000/cell). Only the "TATA" box located at position -29 was used to initiate transcription in vivo. This activity and specificity of in vivo transcription was demonstrated in vitro by employing restriction enzyme truncated cloned CPC-1 and a cell free extract.

To determine the minimal sequences necessary for leukemia induction by MuLV, the genomes of two closely related, recently derived variants which differ in pathogenicity were compared. Two strains of ecotropic, XC-positive MuLV, leuk-1 and leuk-3, were isolated from stocks of rapid leukemia-inducing virus NIH Cl 6 and proviral DNA obtained from acutely infected mouse cells. Linear forms were purified, ligated and cloned. Upon transfection of cloned viral DNA on NIH 3T3 cells, replicating virus was obtained and inoculated into newborn NFS/N mice. Leuk-1 induced leukemia with an incidence of >90% and an average latency period of 2.5 months. Leuk-2 did not cause leukemia during more than 5 months of observation. Comparison of the cloned genomes of leuk-1 and leuk-2 by heteroduplex mapping showed that they were homologous except near the LTR of leuk-1.

Technological advances in somatic cell genetics have permitted the identification and characterization of mammalian cellular loci which participate in neoplastic transformation. The domestic cat and man appear to be choice model systems for biological and environmental mutagenesis but since a substantial biochemical genetic map of the cat was not available, work in this Laboratory has made substantial contributions towards its development and validation. A map of 31 biochemical loci located on 16 feline syntenic (linkage) groups was derived with most linkage groups having been assigned karyologically to one of 19 feline chromosomes. A survey of feral cats revealed 12 polymorphic biochemical loci. Our colony of feral and breed domestic cats has approximately 25 biochemical and/or morphological segregating loci which are amenable to genetic analysis. Over 20 biochemical genetic variants also have been identified which segregate in interspecies sexual crosses between *Felis catus* (domestic) and *Felis bengalensis* (wild). The feline genetic map shares a striking conservation of linkage associations with that of homologous loci previously mapped in man. For the 31 loci located on 16 groups in the cat, the human linkages are nearly identical. The evolutionary implications of this striking concordance are significant since the chromosome organization apparently has maintained some order despite the 80 million years of divergence (between primates and felids). The comparative genetics has valuable predictive value since localization of a gene in the cat strongly suggests the position of a homologous locus in man. This aspect may be especially important for the identification of mammalian genes, retroviral sequences and controlling elements involved in either susceptibility or controlling host-response to the onset and progression of malignancy.

A number of examples of cancer-associated loci have been identified and are at various stages of genetic analysis in their respective systems. Among these are (1) a retrovirus integration site: *BEVI* is a gene located on human chromosome six which provides a high affinity site for integration of baboon endogenous virus; (2) receptors: the feline receptor for epidermal growth factor (EGF) (to be discussed later) is modulated in transformed cells and has been linked to an enzyme gene GSR, provisionally located on feline C2; (3) endogenous viral sequences: the 15+ endogenous RD114 viral integrations in normal cat DNA have been found on a number of feline chromosomes and specific assignment of each using molecularly cloned probes is in progress; (4) cell surface antigens: allogeneic feline antisera has been derived from over 20 reciprocal surgical skin grafts of colony cats. Sexual and parasexual genetic analysis is in progress; (5) restriction genes: *BVR1* is a feline X-linked gene which restricts murine B-ecotropic virus in mouse x cat somatic cell hybrids. *Akvr-1* is a murine restriction gene polymorphic in the feral Lake Casitas mouse population which dominantly restricts viremia and associated leukemia in the AKR mouse.

During analysis of gene-enzyme variation in natural populations of mice, cats and man, it was found that homologous enzymes which were monomorphic in one species were also monomorphic in the other two. Conversely a group of systems (22 of 57 studied) represented a polymorphic cluster in which polymorphism was evident in either two or three of the studied species. These results suggested that the identity of a polymorphic locus is more a function of the gene itself rather than a function of the species.

The basis for tissue tropism for transformation by oncogenic MuLV was detected. Endogenous C3H MuLV was adapted to high titer growth in fibroblast cells in vitro. The most common tumors induced by this virus in vivo, however, were lymphomas rather than sarcomas. The tissue distribution of cell surface receptors required for infection by ecotropic MuLV was determined. Binding experiments with ^{125}I-labeled viral envelope glycoprotein gp70 and cells freshly prepared from all major mouse tissues showed that lymphoreticular tissues had the largest number of free receptors followed by brain and lung. Thus, the number of receptors determined the susceptible target tissue. Yet, xenotropic plaque-purified clonal isolates of rapidly replicating MuLV showed a great variability in the location of the resulting virus-induced lymphoid tumor (thymus, spleen, a single lymph node, liver, kidney or a combination of these tissues). Using clones of hybrids between Chinese hamsters and mouse cells it was determined that receptors for different classes of endogenous mouse type C viruses are coded by independently segregating genes, a chromosome 5-coded gene for one class of ecotropic MuLV, a chromosome 2-coded gene for another class of ecotropic MuLV, and still another gene for receptors used by xenotropic MuLV. In typing the new oncogenic MCF class of MuLV isolated, it was found that several of the leukemogenic isolates used a chromosome 2-coded receptor for infection of fibroblast cells in vitro.

Relatively weak transforming variants of MuLV which induce tumors with a latency period greater than two months are most common. Established laboratory strains of MSV and some of our more recent cell culture-derived isolates transform infected cells instantly to full autonomy of growth. In the animal, these transformed cells have a pronounced tendency for metastasis and release growth stimulating and transforming polypeptides. Most solid tumor-inducing viruses have a latency between 3 and 9 months. Recently, transforming viruses which induce solid tumors within two weeks have been obtained. These isolates behave similarly to laboratory strains of MSV. Some are defective for replication and able to form nonproducer transformed cells.

Transforming viral genomes were isolated from chemically transformed cells chronically infected with endogenous MuLV. These genomes have been molecularly cloned and were examined to define their structure and biological activity.

To test the hypothesis that the virulence of endogenous ecotropic MuLV in inbred mouse populations is a major determinant of leukemia incidence in these strains, it was demonstrated that the difference in infectivity observed between BUDR-activated ecotropic MuLV from the low leukemia mouse strains C3H/He as well as BALB/c and the high leukemia strain AKR correlates directly with a difference in their leukemogenicity. C3H/He MuLV and BALB/c MuLV from nontransformed cells was poorly infectious and induced a 0.5% occurrence of leukemia late in life, whereas ecotropic, XC-positive AKR MuLV-induced a 60-80% incidence of leukemia in inbred, leukemia-negative NSF/N mice. The major biochemical difference between these viruses was within the envelope gene region affecting the antigenicity of the major envelope glycoprotein. The pathogenic MuLV from AKR has a glycoprotein carrying the antigenic determinants of G_{IX} which is absent in the poorly infectious, less pathogenic or nonpathogenic C3H and BALB N-ecotropic MuLV.

B. Identity, Nature and Function of Hormone-like Control and Signal Factors that Mediate the Genetic and Phenotypic Expression of Malignancy in Mammalian Cells.

Perhaps one of the most exciting and promising areas in cancer research at present concerns the discovery of an ever increasing number of factors or families of hormone-like cellular substances that have profound influences on the phenotypic, and probably genotypic, behavior of mammalian cells. They are peptides, generally not systemically produced, generated by cells. Many stimulate growth and mitogenic activity (e.g., epidermal growth factor, EGF), others resemble insulin (insulin-like growth factor, IGF) in their actions, and others (transforming growth factors, TGFs) stimulate cells to phenotypic malignant behavior. These signals act by interacting with specific receptor sites on the cell membrane. The demonstration of "autocrine" stimulation opens a whole conceptual and practical area of research with significant potential applications for control and diagnosis of malignancy. So numerous are the findings in this area, developed in large part in this Laboratory, that only highlights can be mentioned.

A family of transforming growth factors (TGFs) related to, but distinct from, epidermal growth factor (EGF) has been partially purified from the conditioned medium of human tumor cells as well as from extracts of the tumor cells themselves. These biologically active peptides produce at least some of their effects by interacting with EGF-specific membrane receptors. Cells transformed by RNA tumor viruses, as well as an occasional clone transformed by chemical carcinogens, no longer have available EGF receptors. Various other receptor-ligand binding systems are not altered. When human tumors were examined, a small fraction of sarcomas and carcinomas had lost their EGF receptors while retaining their receptors for insulin-like growth factors (IGF). Certain other human tumors, in particular one human fibrosarcoma, lost IGF receptors while retaining EGF receptors. When human tumor cell lines were thus screened for absence of EGF receptors, the lines A673 (rhabdomyosarcoma), 9812 (bronchogenic lung carcinoma) and A375 (metastatic melanoma) were found to release TGFs. Emphasis is being placed on higher production yields, and improved purification and concentration. This includes a 20,000 molecular weight TGF which is produced by each of the three human high-producer cell lines. Using extensively purified human TGF, no conditions were found where the EGF receptor binding activity could be physically separated from the transforming activity leading to the conclusion that TGF is able to bind to the EGF receptor.

Based on findings in this and other laboratories, the theory has been set forth that malignant transformation of cells may be mediated by the release of growth-promoting polypeptide hormones or hormone-like agents such as TGF. This polypeptide(s) is a strong mitogen; causes overgrowth of cells in monolayer culture; causes morphologic transformation of normal cells; and causes anchorage-independent growth (a property in cell culture that correlates best with tumorigenicity in vivo). Furthermore, to account for the observed lesser requirement of transformed cells for exogenous growth factors, TGF is produced by the putative transformed cell itself. The putative transformed cell has its own functional cellular receptors for the polypeptide, allowing phenotypic expression of the peptide by the same cell that produced it. The term "autocrine secretion" has been proposed for this type of self-stimulation, whereby a cell secretes a hormone-like substance for which the cell itself has functional external receptors. An example of this phenomenon is the production of growth factors of the IGF type by the human fibrosarcoma cell line, 8387, in culture. This line, under normal culture conditions, appears to lack available IGF receptors but still has EGF receptors. However, if the cells are plated at low cell density and the medium is continuously perfused to prevent rebinding of the growth factor produced by the cells to the IGF receptors on the same cells, then the 8387 cells can be shown to have specific IGF receptors. The inability to detect IGF receptors under the standard culture conditions results from the rebinding of released peptides to the specific membrane receptors.

Three major peaks of transforming peptides, sarcoma growth factors (SGFs), were found in serum-free supernatant fluids from virus-transformed mouse 3T3 cells. The first consisted of a heterogeneous group of proteins with apparent molecular weights around 20,000; the second sharper and more pronounced peak contained an anchorage-independent growth-stimulating activity with an apparent molecular weight of 9,000-10,000; and the final peak contained a transforming activity corresponding to EGF-competing activity in the 6,000 molecular weight range. Emphasis so far has been on characterizing the 9-10K peak of activity. Antibodies to EGF do not detect SGF in either radioimmunoassays or immunoprecipitation tests. A binding protein from mouse serum that complexes to EGF does not bind to any detectable level to SGF. Nevertheless, both EGF and SGF bind to the 160,000 molecular weight EGF membrane receptor protein, and both bring about the specific phosphorylation of tyrosine residues in the receptor as a result of binding. The most important differences between these growth factors, however, are in their effects on susceptible cells. SGF produces a profound phenotypic alteration in cultured cells and confers on them the ability to behave as transformed cells. The cells are not permanently transformed, however.

Transforming growth factor (TGF) appears also to be produced normally in vivo during embryonic development. Acid-ethanol extracts of 12-13 day-old mouse embryos were found to contain measurable levels of a 10K and 17K TGF that could be readily separated from the 6K EGF which was also present. This suggests that TGF may be normally produced during the course of embryonic development and may have a normal role in the expansion and migration of different populations of fetal cells.

A finding of potentially great importance was the demonstration of epidermal growth factor (EGF) in human urine. Human urine contained EGF (6K) and an 8K variant of EGF which competes for EGF receptors and, unlike the 6K form, supports soft agar growth of cells. EGF was demonstrated in samples taken from normal male and female donors, females in mid- late-term pregnancy and from a male patient with small cell carcinoma of the lung. Comparisons concerning levels of excretion and further characterization of these factors in urine are in progress.

Human TGFs were found to be closely associated with tyrosine-specific protein kinase activity which has been strongly associated with transformed cells. In view of the phenotypic response of fibroblasts to TGF and the report of EGF-stimulated, tyrosine-specific protein kinase activity associated with the EGF membrane receptors, the phosphotyrosine levels in human TGF- and mouse EGF-treated cells were examined. The human tumor line with the greatest concentration of available EGF receptors, A431, exhibited a pronounced increase in total phosphotyrosine in response either to mouse EGF or to TGF (derived from the culture fluids of the A673 human rhabdomyosarcoma cell line). The overall extent of tyrosine phosphorylation in these growth factor-treated cells was comparable to those characteristic of RNA tumor virus-transformed cells. Concentrations of phosphotyrosine in other cell lines, including two lines, A673 and A2058, which themselves are high level producers of TGF, remained unaltered following exposure to either human TGF or mouse EGF. Finally, the already elevated levels of phosphotyrosine in Gardner feline sarcoma virus (G-FeSV)- and Abelson leukemia virus (AbLV)-transformed rat cells were not further increased in response to either growth factor. Further evidence for this concept was derived when phosphorylation of a major protein of around 160,000 molecular weight was observed in EGF-treated, but not in control A431 cells. ^{32}P-labeling of this substrate was observed at EGF concentrations as low as 0.01 μg/ml and reached a maximum at a concentration of 1.0 μg/ml. On the basis of both molecular weight and immunoprecipitation by an antiserum with specificity for the previously described 160,000 M_r EGF membrane receptor, this phosphorylated cellular substrate was indistinguishable from the EGF receptor itself. Thus, the EGF receptor phosphorylation in response to growth factors involves a kinase associated with the receptor. The binding to this receptor of EGF, SGF, or TGF alters its properties in such a way as to result in phosphorylation of the receptor protein. These findings provide a possible connection between the TGFs described here and the various transforming proteins of RNA tumor viruses. Growth factors, like

TGF, that are able, by binding to receptors on the external cell surface, to produce intracellular alterations in various enzyme activities could, by their continued presence, continue to maintain the transformed phenotype.

Another interesting finding with potential clinical applications was the discovery that crude preparations of interferon contain contaminating tumor cell inhibitory factor (TCIF). A group of peptides was found during experiments using human leukocytes that had been stimulated to produce interferon by treatment with Sendai virus. Since the initial steps in the purification of interferon were similar to those used to isolate TGF and ISF it was considered possible to find biologically active factors in such crude extracts in addition to interferons. It was found that, in addition to interferon, a low molecular weight peptide (M_r= 10,000) was isolated that had no detectable interferon activity, yet was a potent inhibitor of the growth of the human melanoma cells. The chromatographic properties of TCIF as well as its biologic properties, allow it to be readily distinguished from both TGF and ISF.

Analysis of epidermal growth factor (EGF) and nerve growth factor (NGF) receptors on human lung cancer cell lines demonstrated potential utility for improving clinical diagnosis and tumor classification. Sixteen human lung cancer cell lines were examined for the presence of specific membrane receptors for EGF and NGF using ^{125}I-radiolabeled growth factor binding assays. Eleven cell lines were derived from patients with small cell carcinoma of the lung (SCCL). Three of these lines were designated as "converters" to non-SCCL after losing SCCL morphology and biochemical properties during passage in vitro. The remaining five cell lines were derived from patients with adenocarcinoma and large cell carcinoma. All 11 SCCL lines lack EGF receptors on their cell membranes while the 5 non-SCCL lines (with one exception) had EGF receptors as do most other human carcinoma and sarcoma cells. In contrast, NGF receptors were present only on SCCL "converter" cells while the remaining 8 SCCL lines and all 5 non-SCCL lines lack NGF receptors. Previous studies have shown that NGF receptors are found on human tumors of neural crest origin including melanomas and neuroblastomas. The absence of EGF receptors on SCCL cells as well as the presence of NGF receptors on SCCL "converters" cells may reflect a unique embryologic origin of these cells compared to non-SCCL. Alternatively, the absence of EGF receptors on SCCL cells may result from the production of an EGF-related peptide by these cells which masks the available membrane receptors. These findings with regard to EGF and NGF receptors on human lung cancer cell lines further demonstrate the fundamental biologic differences between SCCL and non-SCCL and may provide new approaches to the diagnosis and staging of these tumors.

Abelson murine leukemia virus transformation involves loss of epidermal growth factor binding sites. Cells transformed by the Abelson strain of murine leukemia virus (AbLV) were shown to resemble mammalian sarcoma virus transformed cells in that they also exhibited reduced levels of EGF binding. EGF binding was restored to control levels following loss of polyprotein expression in morphologic revertants of AbLV-transformed clones. Similarly, EGF remained at high level in cell lines infected with AbLV td mutants previously isolated in our laboratory and shown to encode polyproteins deficient in tyrosine-specific protein kinase activity. These findings raise the possibility that the AbLV-polyprotein associated protein kinase activity mediates transformation through a mechanism directly or indirectly involving abolition of EGF binding sites.

Certain lipoproteins have also been shown to be stimulators or essential factors of cell growth. This was demonstrated by testing the growth of embryonal carcinoma (EC) cells at low density in defined media. EC cells can be grown at normal cell densities (10^4 cells per cm^2) in defined medium, however, at a density 10- to 20-fold lower most cells do not survive and cell proliferation is very limited. This problem can be overcome by the addition of purified serum lipoproteins to the medium. Each of the three major classes of lipoproteins, high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) have been found to support continuous cell growth in defined media at the lower densities. Serum albumin, a carrier of fatty acids, was also found to significantly improve the growth of EC cells at low density. In this case the effect of fatty acids is clear cut. Although bovine serum albumin is very effective, fatty acid-free BSA is without effect unless fatty acids, such as oleic acid, are also added. In other studies, ethanolamine, an important precursor for several membrane lipids, was found to stimulate the growth of EC cells in medium at low density. Taken together, these findings strongly suggest that certain lipids are important supplements for defined media.

Progress is occurring rapidly along a broad front of growth and transforming factors. Each of the factors described, and others still at preliminary stages, are being produced, isolated, purified, sequenced, and characterized for their biochemical and biological modes of action. Leading candidates with stronger actions implicated in the induction or maintenance of transformation are being selected for scaled-up production by traditional or alternative means. Increased availability of these factors is imperative to permit the most obvious investigations. The fact that many of these important factors are being produced by, and are clearly active on, human cells (both normal and tumor) lends urgency to these efforts to produce factors in large quantities.

C. Identification and Characterization of Similar Factors that Specifically Interfere with the Host's Defensive Physiological Responses to Cancer.

Another explosive area of research concerns the detection of immunosuppressive factors (ISFs) in human tumor cells. ISFs now permit molecular studies into some of the mechanisms through which malignant tumors, even at early stages profoundly affect the host's ability to respond immunologically to the tumor and may give some insights into the phenomenon of metastatic spread. The ISFs were found in crude supernatant preparations of Moloney-MSV-transformed mouse 3T3 cells. Very similar factors were isolated and partially purified from human tumor cells, in particular, from a cloned line of human bronchogenic carcinoma cells. Normal mouse 3T3 cells and normal human diploid fibroblast cell strains release little or no ISF when compared to their transformed counterparts. The major activity from both mouse and human tumor cells is an 8,000 molecular weight peptide that can be readily separated from TGF that is also produced by the same cells. ISF inhibits the response of T lymphocytes to mitogenesis with phytohemagglutinin (PHA) and interleukin I (lymphocyte activating factor) or interleukin II (T cell growth factor). It also blocks the development of cytotoxic T cells and, when injected into mice, the formation of antibodies by spleen cells. Tumor cells, thus, may be able to produce two families of peptides that might be expected to facilitate and protect their ability to proliferate and spread in the body. The first, SGF and TGF, stimulate the growth of tumor cells, including the very same cells that produce TGF. The second family of peptides, ISF, appears to have no effect on the proliferation of fibroblasts and epithelial cells, but is a potent inhibitor of the clonal expansion of T cells and the development of cytotoxic lymphocytes. It is easy to see how such peptides, produced by the tumor cells themselves, could provide the cells with a potent selective advantage that aids in their continued clonal expansion and spread in an increasingly immunologically impaired host.

D. Metabolic Pathways and Intermediate Products Involved in Endogenous Transformation, Tumor Promotion and Cocarcinogenesis.

The Gardner and Snyder-Theilen strains of feline sarcoma virus (FeSV) encode for 115,000 molecular weight (M_r) polyproteins (P115s) as their primary translational products. Cells transformed by an independent variant of Snyder-Theilen FeSV, express an 85,000 M_r protein (P85) which corresponds to the amino terminal two-thirds of Snyder-Theilen FeSV P115. Single tyrosine phosphorylation acceptor sites have been identified within each of these viral gene products and are shown to be phosphorylated both in vivo and by the polyprotein-associated protein kinase activities in immunoprecipitates. These acceptor sites, although highly related, differ significantly. These differences are consistent with the possibility of one or more base substitutions subsequent to the recombinational event(s) leading to acquisition of cellular transforming genes by the feline leukemia virus genome. Each of these FeSV-encoded polyproteins are membrane components and their tyrosine acceptor sites are phosphorylated both in purified membrane preparations and by incubation of intact cells in ATP-containing medium.

It has been further demonstrated that the high molecular weight polyprotein translational product of Snyder-Theilen FeSV has transforming function. Mink cells nonproductively infected by transformation-defective (td) mutants of the Snyder-Theilen strain of FeSV are morphologically nontransformed, fail to grow in soft agar, bind epidermal growth factors (EGF) as efficiently as control mink cells and lack rescuable transforming virus. Although levels of expression of the major viral polyprotein translational product (P85) in td mutant infected clones are comparable to those of wild-type transformants, polyproteins expressed in td mutant clones lack detectable protein kinase activity. Moreover total cellular phosphotyrosine levels are not elevated significantly above control values. Out of a large number of these transformed mink cell clones isolated, the majority were found to revert to a nontransformed morphology upon continuous passage in cell culture. Such nontransformed variants, as well as a Gardner FeSV transformed mink cell revertant clone, lacked detectable polyprotein expression and exhibited levels of phosphotyrosine and EGF binding similar to those of control mink cells. These findings provide strong evidence favoring the involvement of the Gardner and Snyder-Theilen FeSV encoded polyproteins and their associated tyrosine-specific protein kinase activities in the maintenance of transformation.

Differences in mechanisms of transformation by independent FeSV isolates were observed. In contrast to the Gardner and Snyder-Theilen strains of FeSV, McDonough FeSV encodes, as its major translational product, a 170,000 M_r polyprotein with probable transforming function, but lacking detectable protein kinase activity. Total cellular levels of phosphotyrosine remain unaltered in McDonough transformed cells and the major McDonough FeSV polyprotein translational product lacks binding affinity for a 150,000 M_r cellular phosphoprotein, P150, previously shown to specifically bind Gardner and Snyder-Theilen encoded gene products. These findings argue for major differences in the mechanisms of transformation by these independently derived FeSV isolates.

Studies have been conducted to identify the sequential progression of genetic or regulatory changes which occur in mammalian cells when they are exposed to exogenous or endogenous tumor promoters. Results indicate that promotability behaves as a dominant genetic trait independent of whether cells become infected with, or carry within their genes, viral-associated genetic sequences and further that promotability is not a stimulation to proliferation, but rather to metabolic internal shifts affecting other phenotypic controls. Exposure to 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a tumor promoter, switches off procollagen synthesis pre-translationally and probably transcriptionally. This action is reversible by retinoic acid, known to act post-translationally. Phorbol esters produce specific changes in ganglioside biosynthesis. The binding of phorbol diester to cell membrane binding sites can be blocked by phospholipid methyltransferase and other inhibitors. It has been shown further that phorbol esters, which stimulate cell growth in culture and have tumor-promoting activity in vivo, alter the EGF receptor affinity. Exposure of cells in culture to various hydrolytic enzymes and certain lipid-disrupting agents inhibits the binding of EGF to its receptors suggesting a role for membrane phospholipids in the EGF-receptor interaction. Vitamin K3 and related quinones also modulate the affinity of EGF and multiplication stimulating activity (MSA) for their receptors. Phorbol esters cause a rapid release of fibronectin from cells in culture, with concomitant changes in cellular morphology. Diterpene tumor promoters are probably mediated through high affinity receptors for 20-3H-phorbol-12,13-dibutyrate (PDBu). These studies formed a basis for the proposal that TPA and its active analogues probably have some structural resemblance to an endogenous growth-promoting or differentiation modulating substance(s) that has specific membrane receptors. A binding protein for biologically active phorbol and ingenol esters was detected and partially purified from mouse sera. A phorbol-12,13-diester 12-ester hydrolase (PDEH), a critical factor in the susceptibility of skin to the tumor-promoting action of phorbol diesters, has been isolated and characterized from murine and human liver cells.

A preliminary and potentially important related finding with clinical implications is that certain rather common neuroleptic and antipsychotic tricyclic drugs (fluphenazine, chlorpromazine, clopenthioxal, 2-chloroimpiramine, impiramine and the phenothiazines) have been shown in this Laboratory to competitively inhibit the interaction between tumor-promoting phorbol esters and their specific receptors suggesting that these widely used drugs could be tumor promoters.

E. Results of Epidemiological Studies on Human Cancers with Associated Viral Etiologies.

This Laboratory has continued studies on Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) and breast cancer (BC) based on continuing evidence that certain oncogenic viruses probably play at least a highly compromising, if not etiological role, in these diseases. A major finding has been the induction of a frankly malignant lymphoma in a white-lipped marmoset as a result of inoculation and infection with Epstein-Barr virus (EBV), a ubiquitous lymphotropic herpesvirus. EBV was originally isolated from Burkitt's lymphoma patients and has been implicated in NPC and certain non-malignant human diseases, including infectious mononucleosis.

Epidemiologic studies of BL and NPC in the United States and Ghana revealed a correlation between age at diagnosis and the target organs involved with tumor. BL appears to affect the most rapidly growing tissues and there is a higher frequency of abdominal involvement in older patients than in younger patients. The analysis of data from the Burkitt Tumor Project in Ghana indicates a time trend with increasing rates of abdominal tumor and an increasing age distribution over the past 8 years; these findings correlate with an apparent decrease in the incidence of BL in Ghana. Evaluation of data from the SEER Program and the Connecticut Tumor Registry demonstrated an interrelationship between age, race and pathology in more than 1,000 American NPC patients. Well-differentiated tumors were restricted to adult white NPC patients whereas undifferentiated tumors were the rule in black and Chinese American NPC patients as well as children of all races.

Seroepidemiologic studies of EBV immunity in Greenland Eskimos, Danes migrating to Greenland, and Danes living in Denmark indicated an intense exposure to EBV in migrating Danes and suggested that environment plays a particularly important role in determining the EBV pattern of infection in native Greenlanders, who are known to have more than 30-fold higher risk of NPC than Danes.

The IgA antibody to EBV viral capsid antigen (VCA) appeared to provide a useful diagnostic test for NPC. Either high titers or absence of IgA antibody to EBV VCA were of great help in the diagnosis of patients with head and neck cancer and on occasion determined the diagnostic workup of the patient. The radiocomplement-fixation assay detecting antibody to EBV soluble antigen proved even more useful than the IgA VCA test for the monitoring and follow-up of NPC patients. These studies indicated that American BL patients with high EBV titers had a better survival prognosis than patients with low titers; in African BL, antibodies to the early antigen appeared to be useful in predicting late relapse in patients with long periods of remission.

The development of a strong collaborative effort between the National Cancer Institute and the Institute Salah Azaiz in Tunisia has been of great value to provide access to a group of patients with rapidly progressing breast cancer (RPBC). Information gained concerning the etiology and means for control for this form of breast cancer in Tunisia can be expected to help in the control of fulminating breast cancer everywhere. The unusually high frequency of RPBC in Tunisia allows information on this disease entity to be accumulated more rapidly than in the United States. Clinical and pathological data thus far indicate that RPBC in Tunisia is essentially identical to fulminating breast cancer in the United States. The finding of a high content of antigen cross-reacting with murine mammary tumor virus (MMTV) antigens in RPBC has impelled studies regarding the nature of the involvement of this oncogenic virus in at least certain types of breast cancer. The associated chemotherapy studies conducted in Tunisia have already been valuable to the breast cancer patients in those results and are encouraging more American chemotherapists to treat rapidly progressing breast cancer patients which generally were not considered likely candidates for such therapy. The immunologic studies have demonstrated that the immune system in patients with RPBC retains its integrity and thus provides improved guidelines for the management of these patients.

Immunoperoxidase studies performed for the detection of antigen cross-reacting with gp52 of the mouse mammary tumor virus (MMTV) demonstrated significantly more antigen in the tumor biopsies from Tunisian than from American cases. A pilot study in Israel indicated that Tunisian-born Israelis were more likely to have this antigen than European-born Israelis. That the presence of these tumor virus-related antigens in these tumor tissues is significant in the pathogenesis of the disease was supported by a cellular immunity study in which a battery of in vitro assays demonstrated that Tunisians were more likely to develop a strong immune reactivity to MMTV-related and tumor-related antigens than American breast cancer patients, they were less likely to have blocking factor even in the advanced stages of disease, and that Tunisians in general had a stronger pattern of cellular immunity than normal Americans. These findings further strengthen the hypothesis developed in earlier studies that RPBC is actually a hyperimmune disease rather than the result of depressed cellular immunity.

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October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Endogenous Type B Viruses and Cell Components Regulating Cell Division and Cell Configuration

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ursula Heine	Research Microbiologist	LVC	NCI
Other:	R. Gerald Suskind	Medical Director	LVC	NCI

COOPERATING UNITS (if any)

A.E. Demsey, Laboratory of Experimental Pathology, NIAMDA, NIH, Bethesda, MD.

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to determine the role of cytoskeletal and viral proteins in the maturation of a new group of endogenous type B viruses persistent in murine, hamster and marsupial cells. Intracytoplasmic type A particles known to be precursors to such viruses are closely associated with microtubule organizing centers at centrioles in interphase cells and at kinetochores in metaphase-arrested cells. The intracytoplasmic transport of the particles to the cell surface and their release at the cell membrane appears to be dependent on the intact microtubule system of the cell. These results were obtained by studying the intracellular distribution and the mechanisms of intracellular movement of type A particles with a) immunoelectron microscopy (EM) of cytoskeletal proteins, b) quantitative EM of such particles at isolated chromosomes suggesting the transfer of these particles from the cytoplasm to centromeric regions during early metaphase in conjunction with microtubule assembly and c) by employing microtubule inhibitors and correlating morphologic with biochemical events, direct evidence for a major role of the cytoskeleton (microtubules) in the transport and release of type A particles at the cell surface was demonstrated.

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Project Description

Objective:

To identify the precise nature of and molecular basis for the interaction of endogenous type B retroviruses with microtubule organizing centers and cytoskeletal elements of the infected cell.

Methods Employed:

Tissue culture techniques, virus propagation, isolation and quantitation, radioisotopic assays, light and transmission electron microscopy, antibody-mediated labeling, fluorescence microscopy and immunomicroscopy at the level of the electron microscope.

Major Findings:

Recent studies have stressed the importance of the cytoskeleton (actin cables, intermediate filaments, microtubules) for the preservation and regulation of cellular configuration in interphase cells and during mitosis. We have previously shown that intracytoplasmic precursor particles of certain endogenous type B viruses of mammalian origin are closely associated with microtubules and their organizing centers and have thus obtained an excellent model for the study of the possible wider involvement of the cytoskeleton in assembly and maturation of these viruses.

1. The function of microtubules in the intercellular transport of type A particles. In collaboration with Dr. Demsey (Z01 CP 05079-02 LVC), the possible function of microtubules in the transport of intracytoplasmic type A particles from the cytoplasm to the cell surface was examined in NIH 3T3 cells infected with M432 virus by using vincristine sulfate as microtubule inhibitor. Transmission electron microscopy showed in all samples the association of intracytoplasmic type A particles with crystal-like structures of tubulin formed in the presence of the drug. The release of virus at different times after exposure to the inhibitor was quantitated by reverse transcriptase determinations of cell supernatants and by electron microscopy of the number of virions at the cell surface using freeze-dried whole cell replicas. Results of this study indicate a) a temporary inhibition of virus release in the presence of the microtubule inhibitor, b) recovery from inhibition after removal of the inhibitor, and c) a correlation between drug concentration and magnitude of inhibition. These results give evidence of the involvement of the microtubular system in the intracytoplasmic transport of type A particles.

2. Relationship of type A particles to the formation of the mitotic spindle at centriole and chromosome. A method was developed to study quantitatively in the electron microscope isolated chromsomes from mouse and hamster cells during metaphase arrest under conditions that permit concomitant visualization of intracytoplasmic type A particles. In the presence of low concentrations of Colcemid (0.05 μg/ml) migration of type

A particles from the centriole to the kinetochore region of chromosomes was observed in early metaphase. This attachment of virus particles to the kinetochore region was found to be associated morphologically with the continued formation of microtubule fibers at the centriole. During recovery from metaphase arrest type A particles disappear from kinetochores and, quantitatively, an inverse correlation could be established between the number of microtubules nucleating at the kinetochore and the number of remaining type A particles. These results are interpreted as evidence of renewed nucleation of microtubules at the kinetochore region of chromosomes. These findings, together with the observation that type A particles amassed at the distal end of microtubules newly inserted at kinetochores, gives support to the hypothesis that microtubules are necessary in the transport of these viral precursor particles.

3. Immunoelectron microscopy of cytoskeletal proteins in association with migration of type A particles. High resolution electron microscopy, demonstrating changes in the particles' morphology after treatment with vincristine sulfate, suggests that the particles may contain material capable of reaction with the inhibitor in a manner similar to the action of microtubules. Immunoelectron microscopic studies of the distribution of anti-tubulin and anti-actin antibodies in murine cells carrying type A particles were performed. These studies have excluded the presence of these proteins in or at the surface of type A particles. The involvement of microtubule-associated proteins in the attachment of type A particles to microtubules remains to be investigated.

Significance to Biomedical Research and the Program of the Institute:

Although there have been recent fundamental advances, both in the understanding of the organization of the viral genome of retroviruses, as well as of the interaction at the cell surface, little is known about morphologic and immunologic events in the assembly and maturation of such viruses in the cytoplasm. Our studies have demonstrated the involvement of the cytoskeleton, i.e., microtubules and their organizing centers at centrioles and kinetochores, in the assembly and maturation of intracytoplasmic type A particles. Furthermore, they have provided additional data consistent with the hypothesis that these intracytoplasmic type A particles are precursors of type B viruses. The demonstrated relationship of retroviruses to cytoskeletal proteins (microtubules and their organizing centers) suggests new investigative approaches for the study of the modalities of induction of the transformed state in oncornavirus infection, and also points to the possible epigenetic factors in the transmission of these viruses.

Proposed Course:

Extension of these investigations will incude the classical type B viruses (the murine mammary tumor virus) and type D viruses (the Mason-Pfizer virus) since both classes of viruses are preceded in the cytoplasm by type A precursor particles. These studies should provide a basis for a further classification of type B retroviruses.

Publications:

Demsey, A.E., Kawka, D., Galuska, S., Margulies, I., and Heine, U.: Early events of C-type virus budding in cells infected with a Rauscher leukemia virus temperature-sensitive mutant. Arch. Virol. 65: 329-336, 1980.

Heine, U.I., Suskind, R.G., Margulies, I., and Demsey, A.E.: Oncornavirus precursor particles and the microtubule organizing centers. Archiv fuer Geshwulstforschung 50: 715-723, 1980.

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TITLE OF PROJECT (80 characters or less)
Identification of Cellular and Viral Transforming Factors in Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George J. Todaro	Medical Officer	LVC	NCI
Other:	Joseph E. De Larco	Research Chemist	LVC	NCI
	John R. Stephenson	Visiting Scientist	LVC	NCI
	Hans Marquardt	Visiting Scientist	LVC	NCI
	Daniel R. Twardzik	Research Chemist	LVC	NCI
	Stephen A. Sherwin	Clinical Associate	BRMP	NCI
	Michael B. Sporn	Medical Director	LC	NCI

COOPERATING UNITS (if any) R.V. Gilden, G. Schochetman, F.H. Reynolds, S. Oroszlan, T.D. Copeland, Litton Bionetics, Inc. (FCRC), MD; S.B. Mizel, Microbiology Program, The Pennsylvania State University, University Park, PA

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.40	0.60	1.80

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Transforming Growth Factors (TGFs) were isolated from media of human tumor cell cultures and extracts of the tumor cells themselves. The TGFs, including Sarcoma Growth Factor were purified, characterized and their interaction with cell membrane receptors studied. Based on these studies the "autocrine theory" of cell self-stimulation was proposed. TGFs appear during embryonic development and are detected in the urine of pregnant humans and of a patient with small cell lung carcinoma. In additon, a variant of Epidermal Growth Factor (EGF) was detected in urine from normal humans. Human TGFs are closely associated with tyrosine-specific protein kinase activity, while EGF receptors appear to be the substrate for phosphorylation of tyrosine acceptor sites. Immunosuppressive factors (ISF) were identified in sarcoma virus-transformed mouse cells and in human bronchogenic carcinoma cells. A low molecular weight peptide was isolated from crude preparations of interferon that had no interferon activity but inhibited the growth of human melanoma cells. Cells from human lung cancers were examined for specific membrane receptors for EGF and nerve growth factor. Based on the pattern of presence and absence of these receptors, fundamental biologic, and possibly diagnostic, classification of these tumors are seen. The primate type C viruses CPC-1 and MAC-1 were further characterized and classified on the basis of their major internal protein and terminal nucleotide sequences.

Project Description

Objectives:

To investigate the phenomenon of carcinogenesis with special emphasis on delineating the role of oncogenic viruses and virally related genetic sequences in human cancer. Research efforts are conducted on virus-host relationships in viral-induced cancers with emphasis on the detection and characterization of oncogenic viruses, their genetic tracts, and their mode of transmission in animals and man. The host immune mechanism is studied in relationship to the control of viral gene-induced cancers. Investigations are conducted on the molecular processes in carcinogenesis and cocarcinogenesis. Viruses are used as the most precise tools for the analysis of gene control and malignant transformation. Research is conducted to detect viral markers in humans, primates, and other animals. This includes (a) analysis of surface antigens coded for either by cellular or viral genes in normal or malignant cells; (b) detection and characterization of hormone-like factors that may serve as molecular mediators of phenotypic or genotypic malignant expression; (c) characterization of the host immune response to antigens on the surface of malignant cells and RNA viruses; (d) definition of the modes of gene control and transmission of oncogenic viruses and malignancy traits; (e) detection and localization of gene loci involved in pathogenicity, susceptibility, and control of malignancy at the molecular level.

Methods Employed:

Standard cell culture, virological, molecular biological, biochemical, and recombinant DNA technologies are employed. To characterize peptides produced by cell cultures, conditioned medium from cultures maintained in serum-free medium was collected. Gel permeation chromatography, carboxymethyl cellulose and reversed-phase high pressure liquid chromatography (HPLC) were used for purification and characterization of growth-promoting polypeptides. Radioreceptor assays were used to determine competing activities. Soft agar assays were set up in tissue culture dishes containing indicator cells and growth factors. Amino acid sequences of growth-promoting polypeptides were determined by semi-automated Edman degradation. Identification of phenylthiohydantoin amino acid derivatives was made by reversed-phase HPLC and carboxypeptidase digestions. Ion exchange chromatography, gel filtration, high performance gel permeation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and other specialized methodologies were used.

Major Findings:

1. Isolation and characterization of Transforming Growth Factors (TGFs) from human cells. A family of transforming growth factors related to, but distinct from, epidermal growth factor (EGF) have been partially purified from the conditioned medium of tumor cells as well as from extracts of the tumor cells themselves. Conditioned medium from normal cells in culture contains little or no such activity. These biologically active peptides produce at least some of their effects by interacting with EGF-specific membrane receptors. Cells transformed by RNA tumor viruses, as well as an occasional clone transformed by chemical

carcinogens, no longer have available EGF receptors. Various other receptor-ligand binding systems are not altered. When human tumors were examined, a small fraction of sarcomas and carcinomas had lost their EGF receptors while retaining their receptors for insulin-like growth factors (IGF). Certain other human tumors, in particular one human fibrosarcoma, lost IGF receptors while retaining EGF receptors. When human tumor cell lines were thus screened for absence of EGF receptors, the lines A673 (rhabdomyosarcoma), 9812 (bronchogenic lung carcinoma) and A375 (metastatic melanoma) were found to release TGFs. Emphasis is being placed on higher production yields, and improved purification and concentration. This includes a 20,000 molecular weight TGF which is produced by each of the three human high-producer cell lines.

The growth-stimulating factors released by these cell lines were tested for protein concentration, ability to stimulate cells to form colonies in soft agar, and ability to compete with ^{125}I-EGF. A major peak of competition with ^{125}I-EGF binding activity and growth-stimulating activity in soft agar was concentrated by lyophilization and used for further studies. The soft agar growth-stimulation assay is more sensitive than the EGF competition assay. The large form of human TGF (M_r=20,000) (TGF$_L$), at a concentration (10 ng/ml) that gives only a 10% reduction in ^{125}I-EGF binding gives a readily detectable response in the soft agar assay, while control cultures show no response. With the small form of human TGF (M_r=7,000) from the human melanoma line 2058 this phenotypic transformation assay is considerably more sensitive than is the EGF competition assay. If the TGF growth-stimulating effect is mediated by its binding to the EGF receptors, then much less than full receptor occupancy is needed to activate the cell proliferation response. An alternative explanation is that not all of the effects of TGF on cells involve the EGF receptor system. Using extensively purified human TGF, no conditions were found where the EGF receptor binding activity could be physically separated from the transforming activity leading to the conclusion that TGF is able to bind to the EGF receptor.

2. Demonstration of cellular self-stimulation (autocrine) with growth-stimulating polypeptides. Based on findings in this and other laboratories, the theory has been set forth that malignant transformation of cells may be mediated by the release of growth-promoting polypeptide hormones or hormone-like agents. This polypeptide(s) should be a strong mitogen; cause overgrowth of cells in monolayer culture; cause morphologic transformation of normal cells; and cause anchorage-independent growth (a property in cell culture that correlates best with tumorigenicity in vivo. Furthermore, to account for the observed lesser requirement of transformed cells for exogenous growth factors, it should be produced by the putative transformed cell itself, and the putative transformed cell should have its own functional cellular receptors for the polypeptide, allowing phenotypic expression of the peptide by the same cell that produced it. The term "autocrine secretion" has been proposed for this type of self-stimulation, whereby a cell secretes a hormone-like substance for which the cell itself has functional external receptors. An example of this phenomenon is the production of growth factors of the IGF type by the Buffalo rat liver cell line, BRL-3A, in culture. This line, under normal culture conditions, appears to lack available IGF receptors but still has EGF receptors. However, if the cells are plated at low cell density and the medium is continuously perfused to prevent rebinding of the growth factor produced by the cells to the IGF receptors on the same cells, then the BRL cells can be shown to have specific IGF receptors. The inability to detect IGF

receptors under the standard culture conditions results from the rebinding of released peptides to the specific membrane receptors. When an analogous experiment was performed with the human fibrosarcoma line, 8387, the result was the same; IGF receptors could be detected in the sparse cultures. These findings argue that the inability of externally provided ligands to bind to specific membrane receptors reflects the production of growth factors related to EGF and/or IGF. The peptides produced by the cell cultures were isolated, purified and characterized. Three peaks of MSA-competing activity were observed with the largest containing a binding protein for MSA. These growth factors, then, could also play a role in sustaining the proliferation of transformed cells. A comparison of the amino acid sequences of rat MSA and human IGF-II and human IGF-I indicates the rat cell-derived MSA and the human IGF-II differ by only 5 conservative amino acid substitutions; 62 out of 67 residues in the sequences are apparently identical. Each substitution is compatible with a single base change at the DNA level. Based on the extensive amino acid sequence homology, we have proposed the term rat IGF-II for this low molecular weight MSA polypeptide.

3. Characterization of Sarcoma Growth Factor (SGF), another transforming growth factor. Three major peaks of transforming peptides were found in serum-free supernatant fluids from virus-transformed mouse 3T3 cells. The first consisted of a heterogeneous group of proteins with apparent molecular weights around 20,000; the second sharper and more pronounced peak contained an anchorage-independent growth-stimulating activity with an apparent molecular weight of 9,000-10,000; and the final peak contained a transforming activity corresponding to EGF-competing activity in the 6,000 molecular weight range. Emphasis so far has been on characterizing the 9-10K peak of activity. Antibodies to EGF do not detect SGF in either radioimmunoassays or immunoprecipitation tests. The binding protein from mouse serum that complexes to EGF does not bind to any detectable level to SGF. Nevertheless, both EGF and SGF bind to the 160,000 molecular weight EGF membrane receptor protein, and both bring about the specific phosphorylation of tyrosine residues in the receptor as a result of binding. The most important differences between these growth factors, however, are in their effects on susceptible cells. SGF produces a profound phenotypic alteration in cultured cells and confers on them the ability to behave as transformed cells. As the result of a single exposure to SGF, single cells seeded in agar give rise to progressively growing colonies that contain hundreds, even thousands, of cells. The cells are not permanently transformed, however, for when they are reseeded in agar in the absence of SGF they are unable to proliferate. EGF, in contrast, has only a slight effect on monolayer cultures and does not produce large, progressively growing cell colonies in soft agar. While three, or possibly more, different size classes of SGF are produced by the RNA tumor virus-transformed cells, we have never detected EGF production by the transformed cells. We conclude that mouse salivary gland EGF, or a close relative of it, is not the substance activated by the virus transformation. Instead, a different and much more biologically active family of peptides (the SGFs) are produced. When this family is fully characterized it may turn out to have a relationship to EGF similar to that found when IGF is compared to insulin itself.

4. Transforming Growth Factor (TGF) appears to be produced normally in vivo during embryonic development. Acid-ethanol extracts of 12-13 day-old mouse embryos were found to contain measurable levels of a 10K and 17K TGF that could be readily separated from the 6K EGF which was also present. This suggests that

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TGF may be normally produced during the course of embryonic development and may have a normal role in the expansion and migration of different populations of fetal cells.

5. Demonstration of Epidermal Growth Factor (EGF) in human urine. Human urine contained EGF (6K) and an 8K variant of EGF which competes for EGF receptors and, unlike the 6K form, supports soft agar growth of cells. Samples were taken from normal male and female donors, females in mid- late-term pregnancy and from a male patient with small cell carcinoma of the lung.

6. Human Transforming Growth Factors (TGFs) are closely associated with tyrosine specific protein kinase activity. In view of the phenotypic response of fibroblasts to TGF and the report of EGF-stimulated, tyrosine-specific protein kinase activity associated with the EGF membrane receptors, the phosphotyrosine levels in human TGF- and mouse EGF-treated cells were examined. The human tumor line with the greatest concentration of available EGF receptors, A431, exhibited a pronounced increase in total phosphotyrosine in response either to mouse EGF or to TGF (derived from the culture fluids of the A673 human rhabdomyosarcoma cell line). The overall extent of tyrosine phosphorylation in these growth factor-treated cells was comparable to those characteristic of RNA tumor virus-transformed cells. Concentrations of phosphotyrosine in other cell lines, including two lines, A673 and A2058, which themselves are high level producers of TGF, remained unaltered following exposure to either human TGF or mouse EGF. Finally, the already elevated levels of phosphotyrosine in Gardner feline sarcoma virus (G-FeSV)- and Abelson leukemia virus (AbLV)-transformed rat cells were not further increased in response to either growth factor.

7. Epidermal Growth Factor Receptor (EGFR) appears to be the substrate for the phosphorylation of tyrosine acceptor sites. To identify the A431 cell substrate(s) phosphorylated in response to TGF, an in vitro extract labeling procedure, previously shown to result in preferential phosphorylation of tyrosine acceptor sites, was utilized. Confluent cultures of A431 cells were exposed to either human TGF or EGF. Phosphorylation of a major protein of around 160,000 molecular weight was observed in EGF-treated, but not in control A431 cells. ^{32}P-labeling of this substrate, designated P160, was observed at EGF concentrations as low as 0.01 μg/ml and reached a maximum at a concentration of 1.0 μg/ml. On the basis of both molecular weight and immunoprecipitation by an antiserum with specificity for the previously described 160,000 M_r EGF membrane receptor, this phosphorylated cellular substrate was indistinguishable from the EGF receptor itself. Both the A673 human tumor-derived TGF and SGF from supernatant fluids of Moloney murine sarcoma virus (M-MSV)-transformed cells, were analyzed for stimulation of EGF membrane receptor phosphorylation. Exposure of A431 cells to 100 μg/ml of either TGF or SGF resulted in P160 phosphorylation. Other growth-stimulating factors, including phorbol ester derivatives and insulin, tested for stimulation of phosphorylation of the EGF receptor using these assay conditions, had no effect. Heating of TGF under conditions (100°C, 5 minutes) which inactivate protein kinases does not destroy its ability to bind to EGF receptors and does not alter P160-induced phosphorylation. Thus, the EGF receptor phosphorylation in response to growth factors involves a kinase associated with the receptor. The binding to this receptor of EGF, SGF, or TGF alters its properties in such a way as to result in phosphorylation of the receptor protein. These findings provide a possible connection between the transforming growth

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factors described here and the various transforming proteins of RNA tumor viruses. Those growth factors, like TGF, that are able, by binding to receptors on the external cell surface, to produce intracellular alterations in various enzyme activities could, by their continued presence, continue to maintain the transformed phenotype.

8. Detection of Immunosuppressive Factors (ISF) in human tumor cells. The immunosuppressive factors were found in crude supernatant preparations of M-MSV-transformed mouse 3T3 cells. Very similar factors were isolated and partially purified from human tumor cells, in particular, from a cloned line of human bronchogenic carcinoma cells. Normal mouse 3T3 cells and normal human diploid fibroblast cell strains release little or no ISF when compared to their transformed counterparts. The major activity from both mouse and human tumor cells is an 8,000 molecular weight peptide that can be readily separated from TGF that is also produced by the same cells. ISF inhibits the response of T lymphocytes to mitogenesis with phytohemagglutinin (PHA) and interleukin I (lymphocyte activating factor) or interleukin II (T cell growth factor). It also blocks the development of cytotoxic T cells and, when injected into mice, the formation of antibodies by spleen cells. Tumor cells, thus, may be able to produce two families of peptides that might be expected to facilitate their ability to proliferate and spread in the body. The first, SGF and TGF, stimulate the growth of tumor cells, including the very same cells that produce TGF. The second family of peptides, ISF, appears to have no effect on the proliferation of fibroblasts and epithelial cells, but is a potent inhibitor of the clonal expansion of T cells and the development of cytotoxic lymphocytes. It is easy to see how such peptides, produced by the tumor cells themselves, could provide the cells with a potent selective advantage that aids in their continued clonal expansion and spread in the immunologically impaired host.

9. Crude preparations of interferon contain contaminating growth and transforming factors. A group of peptides was found during experiments using human leukocytes that had been stimulated to produce interferon by treatment with Sendai virus. Since the initial steps in the purification of interferon were similar to those used to isolate TGF and ISF it was considered possible to find biologically active factors in such crude extracts in addition to interferons. It was found that, in addition to interferon, a low molecular weight peptide ($M_r = \sim$ 10,000) was isolated that had no detectable interferon activity, yet was a potent inhibitor of the growth of the human melanoma cells. The chromatographic properties of this factor (tumor cell inhibitory factor, TCIF) as well as its biologic properties allow it to be readily distinguished from both TGF and ISF.

10. Epidermal Growth Factor (EGF) and Nerve Growth Factor (NGF) receptors on human lung cancer cell lines. Sixteen human lung cancer cell lines were examined for the presence of specific membrane receptors for EGF and NGF using ^{125}I-radiolabeled growth factor binding assays. Eleven cell lines were derived from patients with small cell carcinoma of the lung (SCCL). Three of these lines were designated as "converters" to non-SCCL after losing SCCL morphology amd biochemical properties during passage in vitro. The remaining five cell lines were derived from patients with adenocarcinoma and large cell carcinoma. All 11 SCCL lines lack EGF receptors on their cell membranes while the 5 non-SCCL lines (with one exception) have EGF receptors as do most other human carcinoma and sarcoma cells. In contrast, NGF receptors are present only on SCCL "converter" cells

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while the remaining 8 SCCL lines and all 5 non-SCCL lines lack NGF receptors. Previous studies have shown that NGF receptors are found on human tumors of neural crest origin including melanomas and neuroblastomas. The absence of EGF receptors on SCCL cells as well as the presence of NGF receptors on SCCL "converter" cells may reflect a unique embryologic origin of these cells compared to non-SSCL Alternatively, the absence of EGF receptors on SCCL cells may result from the production of an EGF-related peptide by these cells which masks the available membrane receptors. Our findings with regard to EGF and NGF receptors on human lung cancer cell lines further demonstrate the fundamental biologic differences between SCCL and non-SCCL and may provide new approaches to the diagnosis and staging of these tumors.

11. Characterization and classification of primate type C viruses. The major internal protein (p30) of MAC-1, an endogenous type C virus of Macaca arctoides and the p30 of CPC-1, an endogenous type C virus of Colobus polykomos were purified and subjected to primary structure analysis. Despite the distant evolutionary relationship (approximately 20 million years) between these two species of Old World monkeys, the amino acid compositions of the viral p30s were very similar and their COOH-terminal sequences (5 residues) were found to be identical. Moreover, the NH_2-terminal sequences (up to 36 residues) differed only in three positions. Both the NH_2- and COOH- terminal sequences showed extensive homology to respective sequences of p30s of known type C viruses from other mammalian species. On the basis of these p30 sequence relationships, MAC-1 and CPC-1 together with MMC-1, an endogenous virus of Macaca mulatta, can be classified into a new subgroup which can be related to the avian reticuloendotheliosis virus and its relatives, transforming viruses of birds. The 5' terminal nucleotide sequence regions of CPC-1 and MAC-1 show a 76% nucleotide correspondence and are 132 and 127 nucleotides long, respectively. Previous strong stop analysis of other type C viruses have defined two subgroups, Rauscher murine leukemia virus/gibbon ape leukemia virus and baboon endogenous virus/RD-114. Based on our sequence analysis of their 5' terminal sequences, CPC-1/MAC-1 form a subgroup. Comparison of CPC-1 and MAC-1 to the sequence of avian spleen necrosis virus (SNV), a member of the reticuloendotheliosis virus group, adds SNV to this subgroup of mammalian type C viruses. CPC-1/MAC-1 and SNV contain conserved regulatory signals in similar positions in their 5' terminal RNA sequences analogous to those observed in other mammalian type C retroviruses. These sequences include the canonical AAUAAA sequence, a palindrome, a putative ribosome binding site, and an integration site. Some of these highly conserved subsequences are common to 3' and 5' terminal noncoding sequences of nonviral eucaryotic messenger RNAs.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of defined polypeptide transforming growth factors suggests that malignant transformation may be controlled some time in the future by means of specific inhibitors of the action of these peptides. With the proposed model of "autocrine secretion", the classic lesser requirement of malignant cells for exogenous growth factors can be simply explained: the endogenous production of growth-promoting polypeptides by the transformed cell lessens its own requirement for an exogenous supply of similar growth factors. The TGF (and SGF) system described above provide considerable experimental evidence in support of the above model. The autocrine concept provides an important conceptual model for certain aspects of malignant transformation and suggests the possibility that

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cells may become transformed by endogenous production of growth factors for which they have their own receptors and to which they are capable of responding. This internal production of growth-promoting polypeptides would serve as a constant stimulus for continued cell division, thereby releasing the peptide-producing cells from some of their normal physiologic controls. The relationship of these factors to the activation of genetic information exhibited by malignant cells will significantly contribute to the definition of the mechanisms and progressive nature of carcinogenesis by either endogenous or environmental factors and circumstances.

Proposed Course:

Expansion of current work on the purification and concentration of transforming growth factors to allow for the collection of enough quantities to permit further characterization and studies. This will include the search for inhibitors to the polypeptide growth hormones for application to the control of malignant transformation. Further characterization of TCIF from human leukocytes. Continuation of studies to define and relate the cellular and viral genetic sequences to the expression and control of carcinogenesis and the host reponse to the onset and progression of cancer.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04822-11 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Multi-Disciplinary Studies on EBV Associated Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul Levine	Medical Director	LVC	NCI
Other:	Sergio Leiseca	Research Biologist	LVC	NCI
	Kamaraju Sreemahalakshmi	Visiting Fellow	LVC	NCI
	Kamarju Sundar	Visiting Fellow	LVC	NCI
	Dharam Ablashi	Microbiologist	LDTV	NCI
	Costan Berard	Pathologist	LP	NCI
	Robert Biggar	Medical Officer	EEB	NCI
	Roger Connelly	Research Statistician	BB	NCI

COOPERATING UNITS (if any) J. Hewetson, Litton Bionetics, Inc. (FCRC), Frederick, MD; F. Nkrumah, Univ. of Ghana, Accra, Ghana; N. Mourali, Inst. Salah Azaiz, Tunis, Tunisia; V. Hyams, Armed Forces Inst. of Path., Washington, D.C.; G. Pizza, Bologna, Italy; D. Viza, Paris, France

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Clinical Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.03	1.60	0.43

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project integrates several disciplines in an attempt to understand the pathogenesis and improve the control of Epstein-Barr Virus associated tumors, particularly nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL). Current projects include the evaluation of data from the American Burkitt Lymphoma Registry and the Surveillance, Epidemiology and End Results Program to characterize BL and NPC in the U.S.; the study of EBV serology in the diagnosis and monitoring of patients with NPC, BL and other EBV-related diseases; development of a primate model for BL, and in vivo and in vitro tests for compounds with activity against EBV.

Project Description

Objectives:

To utilize immunologic and epidemiologic techniques in studies on the etiology and control of Epstein-Barr virus (EBV) associated diseases, with particular emphasis on nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL).

Methods Employed:

Pathology based registries were maintained for American NPC and BL as a source of cases for epidemiologic and laboratory studies. A battery of serologic tests were used to monitor patients with BL, NPC and other EBV-associated diseases. Marmosets and rhesus monkeys were inoculated with EBV and Herpesvirus Saimiri (HVS) and monitored by humoral and cell-mediated immunity (CMI) assays. Transfer factor was prepared from immune leukocytes and the transfer of specific immunity to EBV and HVS antigens was evaluated.

Major Findings:

1. Epidemiologic studies of BL and NPC. Studies on Burkitt's lymphoma (BL) in the United States and Ghana revealed a correlation between age at diagnosis and the target organs involved with tumor. BL appears to affect the most rapidly growing tissues and there is a higher frequency of abdominal involvement in older patients than in younger patients. The analysis of data from the Burkitt Tumor Project in Ghana indicates a time trend with increasing rates of abdominal tumor and an increasing age distribution over the past 8 years; these findings correlate with an apparent decrease in the incidence of BL in Ghana. Evaluation of data from the SEER Program and the Connecticut Tumor Registry demonstrated an interrelationship between age, race and pathology in more than 1000 American NPC patients. Well differentiated tumors were restricted to adult white NPC patients whereas undifferentiated tumors were the rule in black and Chinese American NPC patients as well as children of all races.

2. Seroepidemiologic studies of EBV. Serologic assays applied to a migrant study comparing EBV immunity in Greenland Eskimos, Danes migrating to Greenland, and Danes living in Denmark indicated an intense exposure to EBV in migrating Danes and suggested that environment plays a particularly important role in determining the EBV pattern of native Greenlanders, who are known to have more than 30-fold higher risk of NPC than Danes.

3. EBV assays as clinical tools. The IgA antibody to EBV viral capsid antigen (VCA) appeared to provide a useful diagnostic test for NPC. Either high titers or absence of IgA antibody to EBV VCA were of great help in assisting in the diagnosis of patients with head and neck cancer and on occasion determined the diagnostic workup of the patient. The radiocomplement-fixation assay detecting antibody to EBV soluble antigen proved to be more useful than the IgA VCA test in the monitoring of NPC patients. Serologic studies for EBV associated antigens indicated that American BL patients with high EBV titers had a better survival than patients with low titers; in African BL, antibodies to the early antigen

appeared to be useful in predicting late relapse in patients with long periods of remission.

4. Laboratory studies on the control of EBV. Studies on EBV and HVS in vitro and in vivo were implemented to improve the systems available for the evaluation of antiviral agents in the treatment of BL and NPC. Rhesus monkeys were successfully infected with EBV and a tumor was produced for the first time in a white-lipped marmoset. Transfer factor was prepared from rhesus monkeys immunized with HVS and from an NPC patient in remission with strong cell-mediated immunity against EBV associated membrane antigens. These transfer factors were replicated in vitro using the LDV/7 cell line. The effect of 3 interferon preparations, 2 interferon inducers (poly.I:poly C and poly.ICLC) and cis-platinum (DDP) were studied using a variety of EBV preparations. DDP proved to be the most effective inhibitor of EBV replication.

Significance to Biomedical Research and the Program of the Institute:

The availability of laboratory assays measuring immunity to EBV provides an opportunity to define the factors modifying the effect of EBV in different individuals. Additional diagnostic tools and predictors of morbidity may be developed from these new assays. The IgA antibody to EBV has proven to be an effective diagnostic test for NPC and is now being used frequently for patients in the Washington area. Studies on the interrelationship of pathology, age, racial/ethnic status and EBV serology will help to distinguish among cofactors in the studies on the etiology of NPC. The population based serologic studies in Greenland Eskimos, Greenland Danes and Danish Danes may provide information on the relative importance of environment and genetics as related to the high incidence of NPC in Eskimos. The American BL Registry is providing important information on BL in a non-endemic area, the United States, thus permitting studies on the pathogenesis of the disease without the complicating effect of malaria. Progress on the in vitro and in vivo studies of EBV and HVS will permit the testing of materials potentially useful in the control of EBV-associated tumors.

Proposed Course:

A closer collaboration with participants in the SEER program is expected to provide an expansion of the integrated studies on the pathogenesis and control of NPC in the U.S. Serologic techniques will continue to be applied to populations of normal individuals at high and low risk to NPC. Additional longitudinal studies on patients with BL and NPC will be performed to increase our understanding of which tests will be of value in the monitoring of patients with these tumors. Continued attention will be given to the development of a primate model for BL. All available serologic and CMI assays will be applied to determine which immunologic components correlate with susceptibility and resistance to EBV-associated tumors in nonhuman primates. A number of drugs and biologic modifiers will then be screened by in vitro assays for activity against EBV and these materials will then be applied to in vivo studies of primates infected with EBV. Some of the materials with anti-EBV activity already in clinical use will be evaluated for therapeutic trials in patients with BL.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04825-08 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of the Nature and Control of Feline Endogenous (RD-114-like) Viruses in Cats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Daniel K. Haapala	Microbiologist	LVC	NCI
OTHERS:	William G. Robey	Research Chemist (Biochem.)	LVC	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Virus Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The RD-114 like feline endogenous virus (FEV) is able to productively infect all of more than 20 embryonic feline cells tested. However, the efficiency of infection of cells from different embryos is markedly different varying up to 100-fold. Studies with Moloney sarcoma virus (MSV) serve to divide the embryonic feline lines into two classes, those which transform ("permissive") and those which do not ("restrictive") when only FEV helper virus is provided. However, both "permissive" and "restrictive" cells allow efficient transformation by the FEV pseudotype of MSV when a heterologous helper virus is added. FEV is a complex of viruses. Two isolates derived from the same FEV stock were found to share considerable genetic homology but had unrelated coat antigens. MSV-ml infected S+L- lines were developed from various "permissive" and "restrictive" embryonic cells. One of these was an efficient assay line for numerous cat, mouse, and primate C-type viruses. Additionally, the "restrictive" cell lines provided unique properties which will allow further exploration of the mechanism of cell transformation by mlMSV.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

To characterize and utilize viral nucleic acids and proteins as diagnostic and analytical tools for probing the nature and mechanism of viral oncogenesis. To study different virus-cell interactions to determine the nature of the control mechanism(s) operant in eukaryotic cells.

Methods Employed:

Standard biochemical, biophysical, cell and virus culture methods are used. These include ultracentrifugal, chromatographic, restriction enzymes, Southern transfers, nucleic acid hybridization, gel filtration, electrophoretic, immunological and in vitro protein synthesis techniques.

Major Findings:

1. Growth of FEV in embryonic cat cells. All embryonic cat cell lines tested supported the growth of RD-114-like feline endogenous viruses (FEV) derived from several different sources. The efficiency of productive infection by FEV differed greatly between lines. Studies with the FEV pseudotype of MSV showed that all the lines allowed virus absorption and penetration and therefore the restriction was intracellular.

2. The nature of intracellular restriction. Feline embryonic fibroblast (FEF) cells were cloned and used as targets for FEV in infectious center assays. Five clones examined showed varying sensitivity to FEV. One clone was subcloned and again five clones displayed a range of > 10-fold difference in sensitivity as measured by infectious center assays. All clones and subclones were analyzed and showed normal diploid chromosome compliments eliminating chromosome deletions or duplications as a simple explanation for differing sensitivity.

Moloney MSV was used to generate additional information on the cellular control of FEV. The FEV pseudotype of MSV could productively transform (produce foci in) certain embryonic lines without the addition of exogenous helper virus (e.g. FeLV). These were called "sensitive" lines. Other embryos were transformed foci only with the addition of exogenous helper ("restrictive" lines). However, when the "restrictive" lines were infected with MSV (FEV) and plated on "permissive" cells in infection center assays, we found that a significant proportion of the cells were producing MSV (FEV). It is therefore evident that we can detect virus replication in these cells in the absence of MSV transformation.

3. Construction of S+L- lines from embryonic cells. Both "permissive" and "restrictive" lines of feline embryonic cells were used to construct S+L- cells. We find that the "permissive" S+L- cells are a rapid, sensitive indicator of FEV from several sources. Foci are large and easily read at 5-6 days post infection. However, "restrictive" cells show no foci after FEV infection, although they are transformed by FeLV and murine xenotropic and recombinant viruses.

When "restrictive" S+L- cells are infected with FEV and plated on "permissive" cells, foci are readily detected showing that MSV is rescued from restrictive S+L- cells in the absence of focus induction. It thus appears that either the MSV can replicate in the absence of its transforming function being expressed or that the "restrictive" cell has a special immunity to the transforming function after rescue by FEV.

4. Heterogeneity of FEV. Certain stocks of FEV, when plated on "permissive" S+L- cells, gave two distinct focus morphologies. Both focus types were picked and extensively purified by terminal dilutions. When exposed to anti RD-114 sera, the type 1 virus was neutralized but the type 2 virus was not. Neither was the type 2 virus neutralized by anti-feline leukemia virus (FeLV) serum. However, comparison of the two viruses by RNA cDNA hybridization shows major similarities. Preliminary studies of the proteins from purified virus or pulse-labeled virus producing cells shows that most of the virion proteins and precursor proteins are identical but that the coat glycoproteins are not related.

5. Utility of S+L- FEF cells for assay of heterologous C-type virus. "Permissive" S+L- cells have been used as assays for C-type virus from other species. The cells are at least as sensitive as any other lines available for murine xenotropic and recombinant virions, various RD-114 isolates, and FeLV. We have also been able to detect foci from four different primate viruses, some of which have no other biological assay. To date, attempts to demonstrate focus inducing viruses from human tumor material are negative.

6. Molecular nature of FEV control. We are beginning studies on the molecular nature of FEV control by cat cells. To date, we have been unable to detect differences between the "sensitive" or "restrictive" embryonic cells using a whole virus cDNA transcript from FEV. Both cells (DNAs) show the same pattern of hybridization using a battery of restriction enzymes and the Southern transfer technique and therefore have the same endogenous FEV information.

Significance to Biomedical Research and the Program of the Institute:

The use of viral proteins and nucleic acids as markers in the study of oncogenesis is a basic essential. Increasing the number and availability of such markers has obvious utility in increasing the ability to understand the oncogenic process. The question of how a cell organized vast amounts of genetic material has been and is of a fundamental importance. Cancer causing viruses provide one of several tools which can be used to examine the control of a few specific gene products and so become of general interest. In addition, these studies may prove useful in determining how to control certain cancers.

Proposed Course:

Studies on the control of RD-114 expression in cat cells will be continued. Moloney MSV will be included in these studies to provide the additional capabilities of detecting both early and late viral expression in a clean genetic background. The rescue of MSV from "restrictive" S+L- cells in the absence of transformation provides a powerful tool for studying the mechanism of transformation by m1MSV. Other cat embryo cells and RD-114 virus isolates will be studied to determine if more than two sub-types exist. Additionally, biochemical methods will be employed to further characterize and distinguish the two sub-types isolated to date. Antiserum has been prepared against the coat virion proteins of type-2 FEV and will be used along with DNA-RNA hybridization to determine the possible involvement of type-2 FEV in feline cancers. Molecular studies on the intracellular control of FEV will be continued. These will include the fate of endogenous and exogenous FEV cDNA after de novo infection of "permissive" and "restrictive" cells as well as expression of FEV RNA in each cell type. Types 1 and 2 FEV will be cloned and compared by restriction enzyme mapping. Various restriction fragments including coat-specific DNA sequences will be prepared to continue the above studies.

Publications:

Fischinger, P.J., Blevins, C.S., Frankel, A.E., Tuttle-Fuller, A., Haapala, D.K., Nomura, S., and Robey, W.G.: Reversion of cat cells transformed by MSV: Biological, immunological and molecular properties of flat variant cells. Cancer Res. 41: 958-965, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04826-09 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Expression and Control of Tranformation by Murine Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Peter J. Fischinger	Medical Director	LVC	NCI
OTHERS:	Donald G. Blair	Expert	LVC	NCI
	William G. Robey	Research Chemist (Biochem.)	LVC	NCI
	George F. Vande Woude	Supervisory Research Chemist	LMV	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Virus Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.2	PROFESSIONAL: 0.7	OTHER: 0.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fragments of Moloney murine sarcoma virus (MSV) proviral DNA could be activated to transform cells upon addition of viral long terminal report (LTR) DNA's. The cellular homologue (c-mos) of MSV transforming sequences was not active until LTR's were joined to it in a recombinant DNA clone. MSV-induced tumors in Japanese quail, and sera from regressor birds were examined for antibodies. Viral gene products were altered in various tumor lines. Reversion processes were examined in MSV transformed nonproducing cells.

Project Description

Objectives:

To identify transforming sequences of cloned murine sarcoma virus (MSV) proviral DNA or its normal cellular homologue culture systems. To develop MSV regressor systems in genetically unrelated hosts to study putative anti mos (murine sarcoma virus-specific, transforming nucleotide sequence) antibodies. To identify the nature and mechanisms of reversion from the transformed state.

Methods Employed:

Tissue culture, including propagation and derivation of normal and tumor cultures. Standard virological assays such as focus assay for both transforming and nontransforming oncornaviruses, soft agar assays, isotopic precursor incorporation, isolation and purification of viruses, density gradient centrifugation. Assorted transfection assays with cloned viral or cellular DNA. Isolation and assays of protein and nucleic acid components of viruses and cells including reverse transcriptase assays, nucleic acid hybridization, restriction enzyme analyses, and cloning of DNA fragments. Immunological and biochemical procedures for isolation and induction of immunoglobulins and the purification of various natural host protective factors. Protein and lipoprotein analyses, radioimmune precipitation, immunofluorescent analysis of in vivo and cultured cell samples. Sero-immunoprophylaxis protocols in several in vivo model sytems.

Major Findings:

1. Promotion of proviral DNA transforming capacity. Subgenomic fragments of MSV proviral DNA cloned in pBR322 were used to transfect and transform mouse cells. Upon superinfection with murine leukemia virus (MuLV), some infectious MSV could be recovered if the MSV DNA transfecting fragment consisted of long terminal repeats (LTR) and the MSV specific v-mos (mos from virus, formerly src) nucleotide sequences. Initial analyses are being made of the LTR's of the transforming genome. The virus specific RNA's appear to contain in a single species, not only MSV sequences, but also cloning vector sequences indicating that prokaryotic and oncoviral sequences can be functionally linked. Infectivity of inefficiently transforming MSV proviral DNA segments could be enhanced several hundred fold if separate viral LTR's were added in a cotransfection experiment. At present, if such a separate cloned piece of LTR also contains gag gene derived p60 protein coding information, then a cotransfection with only v-mos cloned MSV DNA, results in transformed cells with immunologically identifiable p60 protein. This approach may help in identifying cells functionally transfected with other functional but nontransforming DNA's of various origins.

2. Activation of normal cell sequences to transforming potential. The normal mouse cell contains a single DNA sequence (c-mos) which is essential identical to v-mos. By itself, the cloned c-mos did not transform cells in transfection, but when LTR's from the 5' end of MSV were linked to c-mos, effective transformation was observed. Clones of c-mos containing LTR's as

well as some intervening normal mouse DNA could also transform cells effectively. The viral LTR, presumed to contain promoter type sequences, was absolutely needed for biological activity. Thus, a viral putative promoter could activate normal inactive cellular genes. Additionally the same promoter could also express some plasmid vector RNA's indicating a broad promotional effect.

3. Rejection of MSV tumors in quail. MSV coated with xenotropic MuLV (B-MuX) could induce sarcomas in Japanese quail, in which both MSV and helper B-MuX replicated. Quails in which tumors regressed were shown to make antibodies predominantly directed against the B-MuX env gene coded gp70, and less against the gag gene of MSV or MuLV, but no antibody capable of precipitating an MSV-coded nonvirion protein. Of special interest was the fact that the MSV gag gene p60 product was found to vary in size, in several MSV yielding quail tumor lines established from individual MSV tumors. The size of precursor polyprotein varied from p54-p58, and the deleted peptides appear to derive from the 3' end of the protein.

4. Reversion of transformaiton. Studies on reversion of MSV transformed cat cells have been terminated with the completion of standard molecular experiments. However, checks of residual mouse LTR sequences will be carried out with DNA probes derived from cloned MSV. Briefly, in revertants isolated from MSV transformed cat cells, no MSV rescue was seen and no MSV-associated products or properties were observed. Molecular analysis with total MSV cDNA or cDNA specific for common or v-mos sequences revealed that MSV had been evicted. Concomitant analysis of feline leukemia virus, or feline xenotropic virus-like sequences showed that these were not obviously altered during the reversion process.

Significance to Biomedical Research and the Program of the Institute:

The cloning of transforming viral genes in bacteria allows DNA sequencing and permits specific physical gene modification to better understand the structure function relationship of such genes. Commitantly, transfection analyses can show exactly which regions are required for biological activity. The protein product can be predicted from the DNA sequence. Two significant elements are salient: 1) Viral promoter regions at the ends of proviral DNA are critical not only for the virus, but also for the promotion of cellular genes; and 2) The normally inactive cellular homologue of a transforming sequence could be activated by viral LTR's. This effect may be extremely useful because one could construct plasmids with desirable nontransforming genes with high degrees of expression of the selected sequence. Construction of specific LTR-marker molecules could serve to readily identify immunologically those rare cells transfected with the sequence of interest. The development of model tumor systems where transforming genes are evolutionarily distinct from the host should be helpful in understanding the tumor rejection process. Finally reversion processes in molecularly defined systems should further elucidate viral and cellular control mechanisms.

Proposed Course:

Studies on the critical transforming sequences of both v- and c-mos as well as other potentially transforming genes will continue. The role of promoter sequences and their localization within the genome will be used to determine whether both upstream and downstream promotions are relevant. Whether promoter insertion results in specific transcription of defined cellular genes will be examined. To identify rare cells efficiently transfected with functioning sequences which do not visibly alter cells, LTR's will be first joined to gag region expression genes and then to sequences of interest, and the cell will be identified with antibodies to the gag gene product. MSV avian regressor tumor systems will be examined further, specifically with the potential of generating nonproductive tumors in quail to identify all reactive antibody populations relative to MSV viral and MSV transformed cell products. Attempts at understanding MSV processing changes will be followed as well as potential inductions of tumors with ts MSV.

Publications:

Blair, D.G., McClements, W.L., Oskarsson, M.K., Fischinger, P.J., and Vande Woude, G.F.: The biological activity of cloned Moloney sarcoma virus DNA-terminally redundant sequences may enhance transformation efficiency. Proc. Natl. Acad. Sci. USA 77: 3504-3508, 1980.

Vande Woude, G.F., Oskarsson, M.K., McClements, W.L., Enquist, LW., Blair, D.G., Fischinger, P.J., Maizel, J.V., and Sullivan, M.: Characterization of integrated Moloney sarcoma proviruses and flanking host sequences cloned in bacteriophage lambda. Cold Spring Harbor Symp. Quant. Biol. 44: 735-746, 1980.

Fischinger, P.J., Blevins, C.S., Frankel, A.E., Tuttle-Fuller, A., Haapala, D.K., Nomura, S., and Robey, W.G.: Reversion of cat cells transformed by MSV: Biological, immunological and molecular properties of flat variant cells. Cancer Res. 41: 958-965, 1981.

Blair, D.G., Oskarsson, M.K., Wood, T.G., McClements, W.L., Fischinger, P.J., and Vande Woude, G.F.: Activation of the transforming potential of a normal cell sequence: A molecular model for oncogenesis. Science 212: 941-943, 1981.

Robey, W.G., Kuenzel, W.J., Vande Woude, G.F., and Fischinger, P.J.: Growth of murine sarcoma virus and murine xenotropic leukemia viruses in Japanese quail. I. Induction of tumors and development of continuous tumor cell lines. Virology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04840-11 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Etiology and Control of Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sergio Leiseca	Research Biologist	LVC	NCI
Other:	Paul Levine	Medical Director	LVC	NCI
	Kamaraju Sundar	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any) J.L. Cicmanec, and E. Lvovsky, Litton Bionetics, Inc. Kensington, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Clinical Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.15	0.90	0.25

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project seeks to study the pathogenesis of virus-associated tumors in man, particularly lymphoma, with an attempt to utilize animal models whenever possible. The studies are designed to provide information as to the possible viral etiology and means of controlling the tumors under evaluation.

Project Description

Objectives:

To evaluate nonhuman primates as models for studies on the etiology and control of human cancer.

Methods Employed:

Ninety rhesus monkeys and six white-lipped marmosets were inoculated with infective Epstein-Barr virus. Rhesus monkeys were studied for their response to an interferon inducer, poly ICLC, in relationship to its effect on EBV and radiation induced immunosuppression. Animals were followed clinically and blood samples were obtained for a variety of virologic and immunologic studies.

Major Findings:

Active infection and tumorigenicity were obtained when primates were inoculated with Epstein-Barr virus. Fourteen of ninety rhesus monkeys inoculated with Epstein-Barr virus (EBV) developed evidence of infection. Of six white-lipped marmosets inoculated with EBV, one developed immunosuppression and a malignant lymphoma.

Significance to Biomedical Research and the Program of the Institute:

The development of a useful animal model for EBV infection in man continues to have a high priority for the Institute. The relative availability of rhesus monkeys and their phylogenetic proximity to humans make this animal a potentially valuable model for EBV infection in man. The production of lymphoma in a white-lipped marmoset (WLM) is important because of the relative availability of this animal for experimental studies.

Proposed Course:

Primates already inoculated with EBV will continue to be monitored for the appearance of lymphoma. Attempts will be made to find other agents increasing the tumorigenicity of EBV in rhesus monkeys and additional animal models will be evaluated.

Publications:

Levine, P.H., Leiseca, S.A., Hewetson, J.E., Traul, K.A., Andrese, A.P., Granlund, D.J., Fabrizio, P., and Stevens, D.A.: Infection of rhesus monkeys and chimpanzees with Epstein-Barr virus. Arch. Virol. 66: 341-351, 1980.

Sundar, S.K., Levine, P.H., Ablashi, D.V., Leiseca, S.A., Armstrong, G.R., Cicmanec, J.L., Parker, G.A., and Nonoyama, M.: Epstein-Barr virus induced malignant lymphoma in a white-lipped marmoset. Int. J. Cancer 27: 107-111, 1981.

Lvovsky, E., Levine, P.H., Bengali, Z., Leiseca, S.A., Cicmanec, J.L., Robinson, J.E., Bautro, N., Levy, H.B., and Scott, R.M.: Stimulation of hematopoietic stem cells by interferon inducers in nonhuman primates receiving fractionated total body irradiation. Int. J. Rad. Oncol. Biol. and Phys., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04868-06 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Generation of New Transforming Mouse Type C. Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ulf R. Rapp	Visiting Scientist	LVC	NCI
OTHER:	Cha-Mer Wei	Expert	LVC	NCI
	Angie Rizzino	Expert	LVC	NCI

COOPERATING UNITS (if any)
Dr. Yuan Fon Lee, Bishop College, Dallas, TX; Dr. Paul O'Donnell and Dr. Nancy Famulari, Memorial Sloan-Kettering Cancer Center, NY

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.85	0.65	0.20

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

New transforming murine type C viruses were isolated and characterized with respect to target cell specificity in vivo, ability to transform cells in culture and their genome structure. Findings included (a) differences in infectivity between leukemia viruses from low and high leukemia mouse strains were directly related to leukemogenicity, (b) the chromosome residence was identified for the receptor-gene code for various classes of viruses, including new oncogenic isolates, (c) a lung carcinoma-associated virus was molecularly cloned, heteroduplex mapping showed a close relationship to spleen focus forming virus (SFFV) in the 3' half of the genome, (d) an attenuated variant of NIH Cl 6, a rapid leukemia inducing murine leukemia virus (MuLV), was isolated after molecular cloning. The genome of this virus was found to be identical with the on cogenic parent MuLV except near the long terminal repeat (LTR), (e) nonproducer transformed cells were isolated after infection with newly derived histiocytoma-inducing virus. The proviral genome from this virus which does not contain the tumor gene of Kirsten sarcoma virus (KiSV) or Moloney sarcoma virus (MoMSV) is presently being purified by molecular cloning.

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Project Description

Objectives:

To isolate and characterize "transforming genes" that have been incorporated into the genome of non-transforming type-C viruses and to employ these new viruses for the development of immunological reagents directed against the products of their transforming genes.

Methods Employed:

Directly transforming retroviruses generally are characterized by the presence of cell-derived, transformation specific sequences that have been incorporated into the retroviral genome. We have developed an in vitro system that has allowed, for the first time, the systematic isolation of new and tissue-specific transforming type-C viruses. We have developed techniques for the derivation of highly infectious forms of these viruses in cell culture. This procedure involves continued selection of the most rapidly replicating progeny virions arising from a series of successive infections of highly permissive host cells. Tumors which develop upon inoculation of mice with such rapidly replicating MuLV were established as cell lines in culture and occassionally were the source of defective transforming virus. Another source was cells chronically infected with MuLV after induction of endogenous type-C virus with IdUrd. Such virus stocks contained minority component viruses with specific toxicity or transforming activity for selected target cells. They could be obtained by cloning virus from the progeny of acute infections of highly permissive cells such as chemically transformed C3H/10T1/2 cells. Tumors that developed upon inoculation of such selected virus stocks into newborn mice were again established in culture and used as a source of specific tumor-inducing viruses. Cloned virus stocks which induced specific tumors were further purified and characterized by molecular cloning in phage vectors. Virus production in cell lines from mice which do not produce type-C virus spontaneously or after treatment with halogenated pyrimidines can be activated by repeated mutagenesis. The most effective mutagen used was ethylnitrosourea (ENU). One genetic change that is commonly observed in MuLV isolates selected for increased oncogenicity in vivo or in vitro is a recombinational exchange in the envelope gene. Such viruses infect cells by attachment to a cell surface receptor that is different from the one used by the parental MuLV. Since some of the recombinants with increased oncogenicity stimulate infected cells to growth in soft agar, we examined this new receptor binding activity to determine whether its presence might be the basis of the mitogenic and transforming activity of the recombinant oncogenic viruses.

Major Findings:

1. Virulence of endogenous ecotropic MuLV as a determinant of leukemia incidence in inbred mice. The first goal was to test the hypothesis that the virulence of endogenous ecotropic MuLV in inbred mouse strains is a major determinant of leukemia incidence in these strains. We demonstrated that the difference in infectivity observed between BUdR activated ecotropic MuLV from the low leukemia mouse strains C3H/He as well as BALB/c and the high leukemia strain AKR correlates directly with a difference in their leukemogenicity. C3H/He MuLV and BALB/c MuLV from nontransformed cells was poorly infectious and

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induced a 0.5% occurrence of leukemia late in life whereas ecotropic, XC-positive AKR MuLV-induced a 60-80% incidence of leukemia in inbred, leukemia negative NSF/N mice. The major biochemical difference between these viruses that we have identified so far was within the envelope gene region and affects the antigenicity of the major envelope glycoprotein of these viruses. The pathogenic MuLV from AKR has a glycoprotein carrying the antigenic determinants of G_{IX}, a differentiation antigen, whereas the poorly infectious, less pathogenic or nonpathogenic C3H and BALB N-ecotropic MuLV is negative for this antigen.

2. Basis for tissue tropism for transformation by oncogenic MuLV. Endogenous C3H MuLV was adapted to high titer growth in fibroblast cells in vitro. The most common tumors induced by this virus in vivo, however, were lymphomas rather than sarcomas. As a first step in analyzing the basis of this tissue tropism for transformation by MuLV, we determined the tissue distribution of cell surface receptors required for infection by ecotropic MuLV. Binding experiments with ^{125}I-labeled viral envelope glycoprotein gp70 and cells freshly prepared from all major mouse tissues showed that lymphoreticular tissues had the largest number of free receptors followed by brain and lung. Thus, we concluded that susceptibility of cells to infection did determine the target tissue for transformation by rapidly replicating MuLV. Yet, preference for transformation of a particular lymphoid cell type may still be a virally determined function. The in vivo experiments with xenotropic plaque-purified clonal isolates of rapidly replicating MuLV showed a great variability in the location of the resulting virus-induced lymphoid tumor (thymus, spleen, a single lymph node, liver, kidney or a combination of these tissues). Using clones of hybrids between Chinese hamsters and mouse cells we previously showed that receptors for different classes of endogenous mouse type-C viruses were coded by independently segregating genes, a chromosome 5-coded gene for one class of ecotropic MuLV, a chromosome 2-coded gene for another class of ecotropic MuLV, and still another gene for receptors used by xenotropic MuLV. In typing the new oncogenic MCF class of MuLV that we isolated, we found that several of the leukemogenic isolates used a chromosome 2-coded receptor for infection of fibroblast cells in vitro. Since some of these envelope recombinants do have the capacity to stimulate infected cells to grow in soft agar it may be possible that some of the cell surface receptors used for infection by MuLV might also function as mitogen receptors. The tissue distribution of such specific classes of receptors might then determine the target cell for transformation by a given recombinant MuLV. We are currently investigating this possibility.

3. Genome comparison of a leukemogenic with a nonleukemogenic variant of MuLV. In order to determine the minimal sequences necessary for leukemia induction by MuLV we decided to compare the genomes of two closely related, recently derived variants which differ in pathogenicity. Two strains of ecotropic, XC-positive MuLV, leuk-1 and leuk-3, were isolated from stocks of rapid leukemia-inducing virus NIH Cl 6 (Rapp and Todaro, 1978). Proviral DNA was obtained from acutely infected mouse cells, linear forms were purified, ligated with Eco R1 linkers and cloned in Eco R1 site of the phage vector λ WES. λ B. Genome size inserts were subcloned in a prokaryotic plasmid vector (PBR 322). Upon transfection of cloned viral DNA on NIH 3T3 cells, replicating virus was obtained and inoculated into newborn NFS/N mice. Leuk-1 induced leukemia with an incidence of ≥90% and an average latency period of 2.5 months. Leuk-2 did not cause leukemia during a

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period of >5 months. Comparison of the cloned genomes of leuk-1 and leuk-2 by heteroduplex mapping showed that they were homologous except near the long terminal repeat (LTR) of leuk-1.

4. Derivation of directly transforming type-C virus from cell cultures. The above observations are relevant only for relatively weak transforming variant MuLV which induce tumors with a latency period greater than two months. Very rapidly transforming type-C virus (tumor formation within weeks) such as the established laboratory strains of murine sarcoma virus (MSV) and some of our more recent cell culture-derived isolates transform infected cells instantly to full autonomy of growth. In the animal, such transformed cells have a pronounced tendency for metastasis and, as was first shown in this laboratory, they produce and release growth stimulating and transforming polypeptides. A variety of solid tumor-inducing viruses were derived from C3H/MuLV in vitro and in vivo which had a latency period between 3 and 9 months. Recently we have obtained transforming viruses which induce solid tumors within two weeks. These isolates behave similar to other laboratory strains of MSV in that some of them are defective for replication and able to form nonproducer transformed cells. Alveologenic lung carcinoma-inducing virus which had a latency of 6-12 months was isolated in vitro by selection of virus with the ability to transform mink lung cells from IUdR-induced C3H/MuLV. Transformed mink lung cells were cloned in soft agar and a particular clone of productively transformed cells was the source of lung carcinoma virus. These cells produce a replicating recombinant mink cell focus forming virus (MCF) class of MuLV and contain, in addition, persistent, unintegrated viral genomes in circular as well as linear forms. Subcloning of these cells in soft agar showed continued segregation of producer and nonproducer transformed as well as revertant cells. We have cloned the unintegrated circular provirus from productively transformed mink lung cells. The genome was cut once by Eco R1 and the permuted proviral DNA was inserted into λ WES.λ B and subcloned in PBR 322. A comparison of restriction maps from this new virus, carc-2, with that from AKR/MuLV showed nonidentity in the entire 3' half of the genome. Comparison by heteroduplex mapping showed a substitution and a deletion loop in the envelope gene. The 5' proximal substitution loop spanned 400 bases and was separated from the second deletion loop of 220 bases by a common region that was 150 bases long. An additional difference from AKR/MuLV was observed near the 3' LTR. Comparison of this genome with that of previously isolated transforming viruses showed no relationship to the tumor gene of Moloney or Harvey sarcoma virus, however, the substitution present in carc-4 was also contained in the genome of spleen focus forming virus (SFFV). The recombinant genome of carc-4 was preexistent as a minority component in the IUdR-induced population of C3H/MuLV since we were able to isolate by molecular cloning an identical genome from this stock.

5. Histiocytoma inducing virus. A virus was isolated with the ability to induce histiocytomas in NFS/N mice after a latency of only 2 weeks. This virus also formed foci in mouse fibroblast cultures and showed a strong toxic component in the case of NIH 3T3 cells or were made up of very large and extended cells in case of C3H/10T1/2. Neither of these cells grew well in soft agar whereas the transforming virus could readily be titered for soft agar colony-inducing units upon infection of epithelial MMCE cells. Using MMCE as target cells we have isolated virus-nonproducer transformed cell clones which contain rescuable transforming genomes. These genomes do not contain the tumor gene present in KiSV or MoMSV. Proviral DNA has been prepared for molecular cloning.

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6. Characterization of tumor cell lines. Cell lines were established from virus-induced lung carcinomas. The target cells were invariably alveologenic type I cells. These cells show some aspects of differentiation in cell culture such as dome formation. Dome formation, which results from the vectorial transport of fluid between the monolayer of cells and the plastic of the dish can be induced in flat cultures by use of 2 mM Dibutyril-cAMP. These findings were obtained by Dr. Angie Rizzino whose work focuses on defining the effects of transformation on the differentiating potential of cells.

7. Cloning of tumor genes from chemically and MuLV transformed cells. This project is performed in collaboration with Dr. Wei. The cells used for this work are C3H/MCA5 and MMCE MB46, which is a MuLV transformed MMCE cell line. DNA from these cells was previously shown to transmit foci upon transfection onto NIH 3T3 cells. Work aimed at the molecular cloning of transforming DNA from these cells is currently underway.

Significance to Biomedical Research and the Program of the Institute:

These studies will facilitate the detection, isolation, and characterization, of new virogenes and oncogenes, and provide knowledge concerning the regulation of their expression and involvement in normal development and malignancy. These techniques are directly applicable to tumor genes of primates and therefore clearly applicable to the research on human cancer.

Proposed Course:

The goal of this research is the isolation of new cell-derived transforming functions from mouse and human cells by use of non-transforming type-C virus. This will be followed by an analyses of the genes coding for such functions after molecular cloning in a phage vector. This will include an investigation of the gene products by immunoprecipitation, generation of monoclonal antibodies and functional analyses. The functional analyses will include assays for protein kinase activity and growth-promoting activity for various differentiated cells in culture. Experience gained in selecting effectively replicating MuLV virions from noninfectious particles will be applied to the task of stabilizing occasionally produced reverse transcriptase-containing particles from human tumor cell lines and selection of infectious human type-C viruses. Adaptation of the technology developed to the selection of primate type-C virus variants that will indicate the presence or availability of virogenes or oncogenes that interact with other viral information and/or chemical carcinogens or co-carcinogens. Characterization of the genome of one of the new histiocytoma-inducing isolates will be accomplished after cloning integrated provirus from nonproducer transformed cells in bacteriophage lambda. The question as to whether a virus-coded transformation-specific protein kinase may be present in the transformed cells will be examined.

Publications:

Keski-Oja, J., De Larco, J.E., Rapp, U.R., and Todaro, G.J.: Murine sarcoma growth factors affect the growth and morphology of cultured mouse epithelial cells. J. Cell. Physiol. 104: 41-46, 1980.

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Keski-Oja, J., Heine, U.I., Rapp, U.R., and Wetzel, B.: Epidermal growth factor-induced alterations in proliferating mouse epithelial cells. Exp. Cell Res. 128: 279-290, 1980.

Rapp, U.R.: Isolation of viruses that induce leukemia, sarcoma and carcinoma from mouse fibroblast cell lines chronically infected with endogenous murine leukemia virus. In Essex, M., Todaro, G.J., and zur Hausen, H. (Eds.): Viruses in Naturally Occurring Cancers. Cold Spring Harbor Conferences on Cell Proliferation. Vol. 7, 1980, pp. 1005-1020.

Rapp, U.R., and Marshall, T.H.: Cell surface receptors for endogenous mouse type c viral glycoproteins and epidermal growth factor: Tissue distribution in vivo and possible participation in specific cell-cell interaction. J. Supramol. Struct. 14: 343-352, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04895-09 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of Polypeptides of Oncogenic Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William G. Robey	Research Chemist (Biochem.)	LVC	NCI
OTHER:	George F. Vande Woude	Supervisory Research Chemist	LVC	NCI
	Peter J. Fischinger	Medical Director	LVC	NCI
	Donald G. Blair	Expert	LVC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Virus Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.9	PROFESSIONAL: 0.8	OTHER: 1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell lines established from Balb-derived xenotropic murine leukemia virus pseudotype of the ml isolate of Moloney murine sarcoma virus (mlMSV BMuX)-induced tumors in Japanese quail showed genetically stable alterations in MSV-specific polyprotein metabolism. Sera from tumored quail and regressor quail contained high titers of type-specific antibodies to BMuX envelope gene determinants, variable titers of antibodies to MSV or BMuX group antigen gene determinants and no detectable antibody to the putative transforming protein of MSV. Permuted envelope gene products of recombinant murine leukemia viruses (MuLV's) were examined by two-dimensional peptide maps. Analogous envelope glycoproteins were found in several virus-free mouse lymphoma cells.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

To identify, isolate and characterize transformation-specific viral polypeptides. Particular attention is given to the immunological detection and biochemical characterization of polypeptides associated with transformation. These methods will serve as tools for examining the molecular events associated with both virus replication and transformation.

Methods Employed:

Viruses were propagated in cell culture. Focus-forming assays, virus neutralization, immunofluorescence and immunoprecipitation methods were employed to determine the presence, extent, and type of virus infection. Specific antibodies to purified virus polypeptides were prepared in goats, rabbits, and quail, and were used to immunoprecipitate cell cytoplasms and mature virions. Immunoprecipitates were analyzed by electrophoresis and two-dimensional tryptic peptide maps.

Major Findings:

1. Isolation of m1MSV variants. We have generated two novel genetically stable variants of the m1 isolate of Moloney murine sarcoma virus, m1MSV, by passage in vivo through Japanese quail. This MSV was previously shown to specify an uncleaved stable precursor, $p60^{gag}$, to the major oncornavirus structural proteins in transformed mammalian cells. When this same MSV was used to transform avian cells, the MSV polyprotein marker was found to have a lower molecular weight and was no longer stable in kinetic experiments. This modification event appeared to happen during tumor formation and once the modification occurred it became genetically stable. Briefly, two cell lines were derived from different tumors, each of which permitted the replication of the Balb-derived murine xenotropic leukemia virus pseudotype of m1MSV, m1MSV(BMuX), with unusually high efficiency. It was further determined that the two isolates specified MSV-specific polyprotein precursors that had different apparent molecular weights than that of the parental m1MSV $pP60^{gag}$. In both the $pP54^{gag}$ and $pP58^{gag}$ variant, preliminary peptide map results suggest that the truncation occurred in the p30 component of the polyprotein precursor. The exact location of the deleted region is under investigation to determine whether the deletion is internal within this region or at the carboxy-terminus of the molecule.

2. Immunological reactivity of sera from tumored quail. One of the original objectives of this project was the generation of antiserum specific to the putative MSV sarcoma gene product (V-mos) through the induction of murine-derived tumors in Japanese quail. That was a reciprocal system to avian sarcoma virus-induced tumors in mammals which yielded antiserum to the avian transforming protein. Sera from tumor-bearing and tumor-regressor quail have been shown to react vigorously with antigenic determinants present on the envelope of the helper leukemia virus. Variable serum reactivity was observed with virus gag proteins and no reactivity was detected against a putative nonvirion MSV vmos gene product. The immune response directed

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against the envelope of the helper leukemia virus was highly type specific both by virus neutralization and immunoprecipitation.

3. Structure of cell surface glycoproteins in murine lymphoma. The role of recombinant MuLV's (RM-MuLV) having env genes derived from sequences of ecotropic as well as xenotropic MuLV's was studied in unusual murine lymphomas of viral origin. Many of these RM-MuLV gp70's differ from those previously described. Analogous gp70's were found on cell surfaces and in supernates of virus-free mouse lymphomas suggesting a direct role of these in leukemogenesis.

Significance of Biomedical Research and the Program of the Institute:

The m1MSV genome which was known to be genetically stable in a variety of mammalian cells was shown to be genetically altered in avian cells. The altered MSV was found to replicate more efficiently in avian cells than in any mammalian cells tested. This may provide insight into the relationship between sarcoma virus polyprotein metabolism and oncogenecity. In studies to be developed, the physicochemical and immunological characteristics of recombinant murine leukemia virus envelope glycoproteins produced by mouse cells derived from radiation-induced lymphomas will be determined. The correlation between the structure of the glycoproteins and biological characteristics of the cells from which they were derived should provide insight into the activation mechanism associated with selective provirus expression by these malignant cells.

Proposed Course:

The studies on MSV-specific polyproteins will be concluded with the determination of the location of the deletion in the gag gene of the two MSV variants described above. Concurrent with the diminished effort in the MSV polyprotein system, increased efforts will be directed to the examination of virus structural proteins (e.g. gp70) present on the surface of leukemogenic cells derived by nonviral induction of the disease.

Publications:

Wood, T.G., Horn, J.P., Robey, W.G., Blair, D.G., and Arlinghaus, R.B.: Characterization of viral specified proteins present in NRK cells infected with a temperature-sensitive transformation mutant of Moloney Murine Sarcoma Virus. Cold Spring Harbor Symp. Quant. Biol. 44: 747-754, 1980.

Fischinger, P.J., Blevins, C.S., Frankel, A.E., Tuttle-Fuller, A., Haapala, D.K., Nomura, S., and Robey, W.G.: Reversion of cat cells transformed by MSV: Biological, immunological and molecular properties of Flat variant cells. Cancer Res. 41: 958-965, 1981.

Robey, W.G., Kuenzel, W.J., Vande Woude, G.F., and Fischinger, P.J.: Growth of murine sarcoma virus and murine xenotropic leukemia viruses in Japanese quail. I. Induction of tumors and development of continuous tumor cell lines. Virology, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04896-09 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of Gene Controlled Events in Neoplastic Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Stephen O'Brien	Geneticist	LVC	NCI
Other:	Mitchell Gail	Medical Statistical Investigator	BB	NCI

COOPERATING UNITS (if any) W. Nash, Litton Bionetics, Inc. (FCRC), Frederick, MD; M.B. Gardner, University of Southern California, Los Angeles, CA; W. Nelson-Rees, University of California, Oakland, CA, and M. Barile, Food and Drug Administration, Bethesda, MD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.34	1.0	2.34

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sexual and parasexual genetic analysis has permitted the detection, characterization and genetic localization of a number of mammalian cellular genes which participate in endogenous type C virus expression. Seven classes of genes have been identified: (1) endogenous cellular DNA sequences homologous to type C viral RNA; (2) integration sites of retroviruses in mammalian chromosomes; (3) growth factor receptors; (4) restriction genes which delimit viral replication; (5) enzyme structural genes; (6) cell surface antigens including major histocompatibility complex (MHC). (7) cellular transforming genes (onc) resecured by defective retroviruses. Examples of each of these gene classes have been detected and mapped in human and feline model systems. The allozyme genetic signature as a definitive characteristic of cultured cells has been applied to monitors of cell contamination, quality control o[illegible] vitro transformation and identification of tissue origin in human e[illegible]onic development. Population genetic analysis of human feline, an[illegible]rine populations has revealed evolutionary conservation of linkage associations and the characters of genetic variance.

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Project Description

Objectives:

1. The construction of a biochemical genetic map of domestic cat (*Felis catus*) with emphasis on genes which relate to the extraordinary viral etiology of leukemia and lymphoma in the species. The specific classes of genes under study fall into seven general groups. The genes include: (1) endogenous cellular DNA sequences homologous to cDNA radioactive probes transcribed from retroviral genomic RNA; (2) chromosomal integration sites for exogenous retroviral insertion and persistence; (3) receptors on cell membranes which interact with virus glycoproteins to determine cell species compatibility and viral host range; (4) restriction genes which delimit virus replication in various animal species; (5) cellular transforming (*onc*, *link*, *src*, etc.) genes; (6) cellular enzyme structural genes; (7) cell surface antigens including antigens homologous to the major histocompatibility complex (MHC) of other mammalian species.

2. The combined application of somatic cell genetics and electrophoretic resolution of cellular DNA sequences following digestion with specific restriction endonucleases to the biology of retroviral integration, excision and transposition in human and feline cells.

3. Genetic analysis of cooperative and sequential gene action in the neoplastic processes and in virogene expression. This consideration involves application of the principles and techniques of microbial genetics to cultured mammalian cells.

4. The development of new approaches to the understanding of genetic control of carcinogenesis. These considerations involve the identification and characterization of genetic targets (cellular genes) of carcinogenesis.

5. Use of inherent genetic variation in human and mouse cells to provide genetic signatures or markers for cells studied in cell culture laboratories. By using allelic isozyme (allozyme) variation in different isolates of human or mouse cells, an allozyme genetic signature of cells can be derived for cell contamination monitors and for approaches to the genetics of development.

6. The genetic analysis of natural populations of man, mice and cats with specific emphasis on populations and cellular genes relating to the epidemiology of certain neoplasias with familial, virological or environmental etiology.

Methods Employed:

The following techniques are employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q) for specific chromosome identification; (4) virological procedures including

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radioimmune assay, reverse transcriptase and viral cloning; (5) immunological assays including radioimmune assay (RIA), cytotoxicity, fluorescent antibody procedures, surgical allograft, and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques including ^{3}H-cDNA transcription in vitro, solution hybridization, HAP chromatography, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis, molecular cloning of proviral circles and preparing clone libraries of cellular DNAs.

Major Findings:

1. Development of genetic analysis of the domestic cat. A preliminary requirement for this effort is the availability of a substantial biochemical genetic map of the domestic cat. Briefly, some 36 different cell hybridization crosses between fresh diploid cat tissue or feline cell lines have generated over 1000 hybrid clones. A genetic map of 31 biochemical loci located on sixteen feline syntenic (linkage) groups was derived. The majority of these linkage groups have been assigned karyologically to one of 19 feline chromosomes. A population survey of feral cats revealed twelve (12) polymorphic biochemical loci. A colony of feral and specific breeds of domestic cat has been established at the NIH Animal Center in Poolesville. The colony has approximately 25 biochemical and/or morphological loci segregating which are amenable to genetic analysis. We have also identified over twenty biochemical genetic variants which segregate in interspecies sexual crosses between Felis catus and Felis bengalensis. Colony cats have been successfully bred into the third generation in a program of mapping a number of genes. The sum of these biochemical genetic characteristics render the domestic cat now a feasible model for genetic analysis.

2. A striking homology of linkage association in cats and man. An unexpected development which occurred during the construction of the feline genetic map was a striking conservation of linkage associations between the feline genetic map and that of homologous loci previously mapped in man. Of the 31 loci placed on 16 groups in the cat, the human linkages are identical with only two exceptions. The first involves a recent human chromosome fusion (human chromosome 2); in this case, the cat resembles the primitive arrangement observed in the Pongidae primates. The second exception is a fusion of two linkage groups (found in primates, human 14 and 15) as a single feline chromosome (feline B3). Certain of the homologous feline and human chromosomes also have apparent G-banding homologies in portions of the homologous chromosomes. The striking concordance of feline and primate genetic maps has two major aspects of biological significance: first, the evolutionary implications are rather significant since the chromosome organization has maintained some semblance of order despite 80 million years of divergence (between primates and felids); and second, the comparative genetics has a predictive value, since once a gene has been located in the cat, a strong suggestion as to the position of a homologous locus in man can be made. This aspect may be especially important in identifying mammalian genes (like retroviruses or controlling elements) capable of transposition during mammalian evolution.

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3. Journal entitled "Genetic Maps" was developed. A correlary to our studies on comparative mapping was the birth of a new journal entitled GENETIC MAPS, editor S.J. O'Brien. This journal is a biannual collection of genetic maps of over 50 microbial, plant and animal species studied in genetics laboratories.

4. Detection and chromosome mapping of mammalian genes with participate in transformation.

As the feline (and human) genetic map was developing, a number of examples of cancer-associated loci have been identified and are at various stages of genetic analysis in their respective systems. Examples of these include: (1) retrovirus integration site: BEVI is a gene located on human chromosome six which is a high affinity site for integration of baboon endogenous virus; (2) receptors: the feline receptor for epidermal growth factor is modulated in transformed cells and has been linked to an enzyme gene GSR, provisionally located on feline C2; (3) endogenous viral sequences: the 15+ endogenous RD114 integrations in normal cat DNA have been found on a number of feline chromosomes and specific assignment of each using molecularly cloned probes is in progress; (4) cell surface antigens: allogeneic feline antisera has been derived from over 20 reciprocal surgical skin grafts of colony cats. Sexual and parasexual genetic analysis is in progress; (5) restriction genes: BVR1 is a feline X-linked gene which restricts murine B-ecotropic virus in mouse x cat somatic cell hybrids. Akvr-1 is a murine restriction gene polymorphic in the feral Lake Casitas, California mouse population which dominantly restricts viremia and associated leukemia in the AKR mouse. This gene is not specifically mapped, although allelism with a number of known murine restriction genes (FV-1, Fv-2, etc.) has been excluded.

5. The genetic analysis of the human BEVI locus. A human locus, BEVI (for baboon endogenous virus integration), has been mapped to human chromosome six and is required for successful infection of rodent x human hybrids with baboon endogenous virus. Molecular hybridization experiments with cDNA probes prepared with baboon virus to cellular DNA of $BEVI^+$ and $BEVI^-$ hybrids of infected human x hamster crosses have demonstrated that BEVI is a high affinity integration site for baboon endogenous virus in the human genome. The data to date, however, do not formally exclude the interpretation that BEVI encodes a function which is required to maintain integration of baboon endogenous virus on other human chromosomes. We have localized BEVI to the short arm of chromosome 6 in a region where GLO1 and human HL-A are also located. The possibility that BEVI is subject to allelic exclusion was further tested by infecting cells heterozygous at two allozyme loci (GLO1 and PGM3) linked to BEVI, on chromosome six. Cloned derivatives of infected cells were fused to mutant rodent cells and derivative hybrids were assayed for BEVI in hybrids containing either maternal or paternal chromosome six homologues. Both hybrid classes were virus-positive, indicating a lack of allelic exclusion of BEVI and, rather, dual integration into both chromosome six homologues in clones of infected human cells. Restriction enzyme analysis of cloned, infected human cells using molecular clones of baboon endogenous virus as

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a probe indicated that integration of human cells takes place in limited numbers per cell (1-2/haploid genome), but at multiple sites. Nonetheless, integrated provirus does indeed segregate with chromosome 6 (BEVI) in hybrid secondary clones. Our results suggest that multiple sites of integration exist on the short arm of chromosome six and these may serve as an array of chromosomal targets of retrovirus integration.

6. Isozyme and allozyme genetic signatures as tool for human genetic analysis. The allozyme genetic signature of human cells (composite allozyme phenotype at eight gene-enzyme systems that are polymorphic in human populations) has found human cell contaminants that have been identified from materials submitted for fetal versus maternal cells for purposes of genetic counseling. The triploid and androgenetic origin of partial and complete hydatidiform moles respectively has been achieved. These carcinomas develop in pregnant women and represent abnormal fetal development. Twelve isozyme systems have been resolved in cultured mycoplasmas and in mycoplasma-infected cells. The systems are useful in identification and discrimination of different mycoplasmas as well as indicators of mycoplasma contamination of cultured cells.

7. Comparative population genetics of mammalian species. In previous years, we have undertaken population genetic surveys of natural populations of mice, cats and man for reasons which related to the neoplasia incidence in these populations. During the analysis of gene-enzyme variation of these species, it became apparent that homologous enzymes which were monomorphic in one species were also monomorphic in the other two. Conversely a group of systems (22 of 57 studies) represented a polymorphic cluster in which polymorphism was evident in either two or three of the studied species. These results suggested that the identity of polymorphic locus is more a function of the gene than of the species. The prevailing hypotheses for classifying polymorphic and monomorphic loci in terms of physiological and physical enzyme characteristics have been reexamined and found to be lacking.

Significance to Biomedical Research and the Program of the Institute:

The characterization of identified loci which participate in cell transformation has two important applications: (1) as the raw material for the dissection of developmental genetic analysis of the cellular events which lead to neoplastic transformation, and (2) as possible targets for carcinogens in screening protocols. The specific understanding of the developmental genetic cascade which characterizes the neoplastic event is necessary for any meaningful attempt to correct and to destroy cancerous tissues. A comprehensive genetics program, from the molecular to the biological species level holds promise in the ultimate resolution of the neoplastic process in man.

Proposed Course: None

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Publications:

Rice, M.C., Gardner, M.B., and O'Brien, S.J.: Genetic diversity in leukemia prone feral house mice infected with murine leukemia virus. Biochem. Genet. 8: 915-928, 1980.

Nelson-Rees, W.A., Hunter, L., Darlington, G.H., and O'Brien, S.J.: Characteristics of HeLa strains: permanent vs. variable features. Cytogenet. Cell. Genet. 27: 216-231, 1980.

Gardner, M.B., Rasheed, S., Pal, B.K., Estes, J.D., and O'Brien, S.J.: *Akvr-1*, a dominant MuLV restriction gene segregates in leukemia-prone wild mice. In Yohn, D.S., Lapin, B.A., and Blakeslee, J.R. (Eds.): *Comparative Leukemia Research 1979*. New York, Elsevier/North Holland, 1980, pp. 149-150.

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Harris, N.L., Gang, D.L., Quay, S.C., Poppema, S., Nelson-Rees, W.A., and O'Brien, S.J.: Contamination of Hodgkin's disease cell cultures. Nature 289: 354-356, 1981.

O'Brien, S.H., Simonson, J.M., Grabowski, M.W., and Barile, M.R.: A survey of isozyme expression in mycoplasmas and mycoplasma infected mammalian cell cultures. J. Bacteriol., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04916-05 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Organization and Evolution of Endogenous Mammalian Type C Viral Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Tom I. Bonner	Expert	LVC	NCI
OTHER:	Edward J. Birkermeier	Staff Fellow	LVC	NCI
	George E. Mark	Expert	LVC	NCI
	George J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The distribution within primates of nucleic acid sequences related to endogenous retroviruses and their sequence organization was studied. Specific goals are (1) to detect and characterize the virus-related sequences in primates and the factors involved in their evolution; (2) to develop probes appropriate for the detection of virus-related sequences in human cells; (3) to characterize the organization of the 50-100 copies of endogenous sequences through restriction mapping and recombinant DNA techniques.

PHS-6040
(Rev. 10-76)

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Project Description

Objectives:

To characterize the distribution within primates of nucleic acid sequences related to endogenous retroviruses, their sequence organization and its relationship to their biological activity. Specifically, (1) to detect and characterize the virus-related sequences in primates and the factors involved in their evolution; (2) to develop probes appropriate for the detection of virus-related sequences in human cells; (3) to characterize the organization of the 50-100 copies of endogenous sequences through restriction mapping and recombinant DNA techniques.

Methods Employed:

The existence of endogenous viruses and their related sequences in many primates indicates that similar viral genomes or related sequences may exist in human cells. Since we would ultimately like to characterize such human viral sequences we have focused on the viruses isolated from man's closest relatives, the Old World monkeys. These viruses include two distinct classes of type C virus: (1) the various, closely related isolates of type C viruses from several species of baboon (we use the M7 isolate) and (2) a group of closely related viruses, including MAC-1 from stumptail macaque, MMC-1 from rhesus monkey and CPC-1 from colobus. Using recombinant DNA techniques we have molecularly cloned unintegrated CPC-1 viral DNA. This DNA and various restriction fragments of it as well as similarly cloned M7 viral DNA have been used as probes to study the organization of related sequences in several primates. We have also molecularly cloned portions of several copies of CPC-1 sequences from colobus cellular DNA in order to characterize the adjacent non-viral (flanking) sequences. Finally we have cloned virus-related sequences from chimpanzee, the closest living relative of man. The chimpanzee sequences have been used to try to detect related sequences in man. In addition because they give another branch of the phylogenetic tree of primate type C viruses they allow to more accurately define the evolution of these viruses.

Major Findings:

1. The organization of CPC-1 sequences in colobus. We have molecularly cloned the complete CPC-1 virus as three non-overlapping EcoRI restriction fragments and generated a nine enzyme restriction map of the cloned DNA. The vast majority of the circular forms of the unintegrated viral DNA have a single rather than a double copy of the long terminal repeat sequence. Restriction enzyme analysis of the approximately 100 copies of CPC-1-related sequences in colobus DNA demonstrate that the majority of these copies have the same restriction sites as the cloned viral DNA. Eighteen of nineteen restriction sites are highly conserved in the cellular sequences. Furthermore the sequences exist without any substantial insertions or deletions. The restriction analysis of the cellular DNA revealed major bands in some enzyme digests, especially EcoRI and Bam HI, which could not be accounted for by the viral map. The intensity of these bands indicated that there was a substantial number of copies of these fragments. A possible explanation of these

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bands could be that they result from the viral sequences being clustered with a repeated spacer sequence between copies. This sequence organization would be analogous to that of the ribosomal and histone genes and would provide a mechanism such as unequal crossing over for maintaining homogeneity among the copies. However analysis using probes from specific regions of the CPC-1 viral genome indicated that the observed bands have homology with the middle of the genome but not the ends. Thus they do not result from fragments which span the viral-spacer junction. Furthermore use of the specific probes reveals that most of the fragments which span the junction between viral and cellular sequences are different from each other. Finally, hybridization of the flanking cellular sequence from a cloned viral-cellular junction fragment to two other such cloned DNAs shows no flanking sequence homology. Thus, there is no support for the hypothesis that the viral copies are organized in a clustered fashion with a common spacer sequence. The nature of the unexpected EcoRI and Bam HI bands was defined by using cloned sequences of chimpanzee cellular DNA (described below) which are related to CPC-1. When identical digests of colobus DNA are hybridized to these chimpanzee sequences and to CPC-1 DNA, the bands predicted from the CPC-1 map and those not predicted by the map show dramatic changes in relative intensity. This indicates that there is a second distinct family of sequences in colobus DNA which is related to CPC-1 but divergent enough from CPC-1 that they are detected more easily by the chimpanzee sequences. Furthermore these sequences are not detected by M7 probes which detect an entirely different set of restriction fragments. Thus, colobus DNA contains at least three distinct sets of type C virus-related sequences.

2. The cloning of virus-related sequences from chimpanzee: We have molecularly cloned, from chimpanzee DNA, sequences which are related to both CPC-1 and M7 viral DNAs. One clone in particular represents a nearly complete viral genome because it contains a terminal repeat structure separated by 8 kilobase pairs of sequence which by heteroduplex analysis is colinear with CPC-1 DNA. Since other primates, such as colobus, baboon and rhesus, contain at least two distinct sets of viral sequences, one related to M7 and one related to CPC-1, we were surprised to find that chimpanzee DNA contains only a single family of sequences related to both viruses. The chimpanzee viral sequence must therefore represent either (1) a recombination of CPC-1 and M7 related sequences for which heteroduplex evidence is negative or (2) a descendent of the common ancestor of M7 and CPC-1.

3. Detection of endogenous viral sequences in man. Since the chimpanzee sequence represents both the M7 and the CPC-1 related sequences in chimpanzee and since chimpanzee is the closest living relative of man, these sequences might be expected to be the best possible probe for the detection of endogenous viral sequences in man. Using these sequences as probes for low strigency Southern hybridizations, we can easily detect related sequences in gorilla, baboon, rhesus, colobus and even mouse DNAs. However we cannot detect related sequences in man, gibbon or orangutan. Clearly, these apes have either never had these sequences or they have lost them. It is highly improbable that the sequences were present in the human-chimpanzee ancestor and have

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diverged rapidly enough since the human-chimpanzee that they escape detection. Such a hypothesis would require that the divergence rate be at least 15 times as great as the rate of divergence of single copy cellular sequences. This in turn would be an order of magnitude faster than estimates of the rate of change for nucleotide substitutions which are of neutral selective value. Thus, these sequences are not essential or even advantageous in an evolutionary sense nor are they closely related tc any normal genes which are essential. Having demonstrated that viral sequences are not present in man using viral sequences from the closest possible relative of man, we are left only with the long shot that human viral sequences might be detected using a viral probe from some more distantly related species. In this case there is no obvious basis for expecting one virus to be more successful than any other.

4. Mapping conserved regions between distantly related retroviruses. Another approach to looking for viral sequences in other species is to use probes from especially conserved regions of the viral genome. In order to define these regions we have used cloned viral DNAs from distantly related retroviruses (AKR, CPC-1, M7). By restricting these DNAs with various enzymes whose sites have been mapped, fixing these fragments on a Southern blot and hybridizing to nick-translated viral DNAs, we can define those fragments which have the greatest sequence homology. The results of such experiments localize the most conserved region to the middle of the viral genome, primarily in the polymerase gene region.

Significance to Biomedical Research and the Program of the Institute:

Our data shows that the pattern of endogenous primate viruses is not as simple as we had previously expected. For sequences related to M7 and CPC-1, there are at least three distinct families in colobus DNA, one in chimpanzee DNA and none in human DNA. The absence of these sequences in human DNA indicates that they play no essential role in cells although there may be essential genes which are too distantly related to detect.

Proposed Course:

Although the viruses we have discussed do not detect any human endogenous sequences, they provide a useful system in which to study the general characteristics of endogenous viruses. The cloned DNA from two viruses plus virus-related sequences from two cellular genomes provide us with the reagents to define the evolution of this system and factors governing their biological activity. A major question concerning their evolution is how long have they been endogenous in the primates. We should be able to determine whether the flanking cellular sequences are the same from one species to another. Since it has been shown by several groups that the retroviruses integrate more or less randomly in the cellular genome, this should provide a good test for their presence in the genomes prior to the divergence of the species being compared.

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Publications:

Bonner, T.I., and Todaro, G.J.: The evolution of baboon endogenous type C virus: Related sequences in the DNA of distant species. Virology 103: 217-227, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04924-08 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Serologic Analysis of Cellular and Viral Proteins with Transforming Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gary J. Kelloff	Senior Surgeon	LVC	NCI
OTHER:	Fulvia Veronese	Visiting Fellow	LVC	NCI
	John R. Stephenson	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

F.H. Reynolds and W.J.M. Van de Ven, Litton Bionetics, Inc. (FCRC), Frederick, MD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 2.1	PROFESSIONAL: 0.9	OTHER: 1.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has clearly and extensively defined the host's immune response to chronic stimulation with endogenous viral antigens which are present in spontaneously occurring tumors and are immunologically active as transplantation antigens. The purification and characterization of these antigens are complete resulting in highly specific reagents. These reagents have made possible studies resulting in conceptual advances of our understanding of how the immune responses of the host interact with each other and the transplantation antigens in determining the outcome of the tumor host relationship. The antigens that have been completely studied thus far have been endogenous viral structural proteins. The transforming proteins encoded by the virus, but clearly of host origin, provide potential transplantation antigens that will understandably be of direct importance in human cancer. The project is now focusing on the production of monoclonal antibodies to these proteins that will result in: the evaluation of these proteins as transplantation antigens, a determination of their presence in tumors throughout the phylogenetic scale, and a better understanding of the mechanisms of transformation and thus the means to biochemically or immunologically alter the events that lead to cell transformation.

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Project Description

Objectives:

1. Production of hybridomas made from immune lymphocytes obtained from rats immune to syngeneic tumor cell lines containing the polyprotein of the feline sarcoma virus(es) or the Abelson leukemia virus. The hybridomas are to provide a library of high titered monospecific antibodies to the different antigenic determinants of the transforming proteins. Immunoepidemiologic characterization of rats bearing tumors produced by various etiologic agents for evidence of antigens reactive with monospecific antibodies to the different antigenic determinants of the transforming proteins. Extension of this survey to tumors obtained from species throughout the phylogenetic scale. Examination of the cell surface reactivity of the monoclonal antibodies with transformed cells and use of such antibody in immunotherapeutic experimentation.

2. Characterization of the naturally-occurring cell-mediated and humoral immune responses to the structural and nonstructural gene products of the endogenous type C viruses. Examination of the interactions of the naturally-occurring humoral and cell-mediated immunity to type C virus containing tumor cells in the aging mouse and its implications for natural tumorigenesis.

Methods Employed:

Cell culture techniques include maintenance of myeloma lines, cell fusions of myeloma cells with immune lymphocytes, cell cloning, and production of monoclonal hybridomas. Immunological procedures include humoral and cell-mediated cytotoxicity assays, blocking assays, immunoprecipitation and gel electrophoresis.

Major Findings:

1. Naturally occurring humoral immunity to endogenous xenotropic and amphotropic type C virus in the mouse. Natural humoral cytotoxic antibodies from 13- and 18-month old BALB/c mice showed a virus-specific complement-dependent activity against target cells nonproductively infected with xenotropic, amphotropic or ecotropic type C viruses. The cytotoxic activity was the lowest against ecotropic virus-shedding cells. Serum obtained from mice of less than 12 months of age had no such reactivity. The cytotoxic reactivity was found to reside solely in the immunoglobulin M fraction which yielded reactivity comparable to the unfractionated sera concerning both titer and relative reactivities to the target cells infected with different type C viruses. Examination of hyperimmune mouse serum from individual normal BALB/c mice of 18 months of age revealed that 80-90% of them were cytotoxic against virus nonproducer mink target cells expressing gp70 or gag gene product. Absorption of sera from 18-month old normal BALB/c mice with cells shedding Class II or Class III xenotropic virus, amphotropic virus, Rauscher MuLV, or Class I murine leukemia virus indicate a closer amphotropic FMR viral subtype specificity of the natural cytotoxic immune response as compared to the amphotropic-xenotypic or the amphotropic-ecotropic specificity. The incidence and the level of

measured humoral cytotoxic activity was sustained in the tumor-bearing animal up to 28 months of age as compared to the background established in the 18month old animal. However, in the nontumor-bearing animal, the incidence and level of cytotoxic reactivity decayed rather rapidly with aging. The sustained cytotoxic reactivity of the serum from old-aged tumored mice might be involved in the progression of the tumor.

2. Natural killer cell activity and humoral blocking activity to endogenous type C viruses of the mouse: Interaction of these two immune responses in the aging mouse and its implications for natural tumorigenesis. A naturally occurring cell-mediated immune response to endogenous xenotropic and amphotropic type C virus occurs in the normal aging BALB/c mouse. This activity is first measurable at about 12 months of age, appearing subsequent to antigenic or infectious expression of the endogenous type C viruses. Concomitant with the appearance of virus specific natural killer cell activity, a virus specific humoral immune response appears and is measurable by the host sera's ability to block the natural killer cell activity in in vitro assays. The blocking activity of this sera is removed by absorption with Class II xenotropic virus (X2V) or Rauscher leukemia virus (R-MuLV). Normal aging mice appear to have the blocking activity of their sera exclusively in the IgM fraction; however, sera from hyperimmunized mice and goats revealed that IgG can mediate the blocking activity as well. Unlabeled concentrated R-MuLV or X2 virus was shown in inhibition assays to effectively compete with labeled target cells for the natural killer cell activity. The nature of the target antigens of the natural killer cells was found to be the products of both the env and gag genes, as shown by the complementary blocking activity of anti-R-gp70 and anti-R-p12 and by the activity of NK cells against mutant virus infected target cells producing only env gene products or only gag gene products. An examination of natural killer cell activity and blocking activity was made in normal and spontaneous tumor bearing mice aged 18 to 28 months. Both NK cell activity and blocking activity were found to be sustained in tumored animals up to 28 months of age; however, the blocking activity, and to a somewhat lesser extent, the NK cell activity decayed in aging nontumored animals after 18 to 22 months of age.

Since the humoral blocking activity dropped rapidly in normal aging animals, but was sustained in tumor bearing animals, sequential monthly test bleeds were performed on large groups of aging mice to determine wether the sustained blocking activity preceded or followed the histological evidence of tumor. The NK cell activity was also examined in each individual animal and while this assay could not be performed serially on the same animal, its measurement at scheduled sacrifices and in tumored animals gave evidence of the trend of this activity. Evaluation of this large sampling of experimental mice clearly showed that the sustained humoral blocking activity preceded the histological evidence of tumor. The NK cell activity appeared later than the humoral activity but in most instances was also detectable prior to development of histologic evidence of tumors in individual animals.

3. Production of monoclonal antibodies to viral proteins. With the goal of producing monoclonal antibodies to the transforming proteins and the

structural components of the virus, syngeneic cell lines have been derived from embryonic cells of the inbred Fisher rat so that the host's immune response could be examined against such cell lines nonproductively transformed by certain type C virus(es). These viruses encode as one of their major translational products, polyproteins with transforming activity. Such proteins are presumably of host cell origin. Cell lines under study include nonproducer cells transformed by feline sarcoma virus (FeSV), Abelson leukemia virus (AbLV) and BALB/c sarcoma virus (B/c-MSV). These various nonproducer clones differ in the types and levels of the gag structural proteins that are produced, but most lines under study have the transforming proteins. Animals hyperimmunized with nonproducer transformed cells are able to regress the syngeneic tumor challenge and antisera obtained from such animals have been shown by polyacrylamide gel electrophoresis to be reactive with the structural and nonstructural portions of the polyproteins. Those antiserum reveal that the polyprotein is immunologically active at the cell surface in viable cells as determined by a chromium release cytotoxicity assay. These regressor rats have also been shown to have immune lymphocytes specifically reactive with target cells containing the polyproteins. The determination of the specific antigen determinants of the polyprotein that are immunologically active at the cell surface will await the development of monoclonal antibodies reactive with these specific determinants. Stable hybridoma cell lines have been prepared by polyethylene-glycol fusion of both the Y-3 and the NS-1 myeloma cell lines with immune lymphocytes obtained from hyperimmunized rats. The immunoglobulin products of these hybridomas are now being characterized and evaluation of the most optimal methods of producing reactive hybridomas are in progress.

Significance to Biomedical Research and the Program of the Institute:

This effort has contributed significantly to our understanding of the host's natural immune response to its spontaneous tumors and to the viral gene products ubiquitously expressed in these tumors. Independent of etiologic relationships, these viral gene products act as transplantation antigens in the tumors. The availability of transplantation antigens whose structural specificities are defined have heretofore not generally been available which has slowed the progress of tumor transplantation research. These studies have clearly established that the host responds to high dose, chronic exposure to an endogenous antigen with an IgM response which by blocking the host's cell mediated NK activity clearly acts to enhance the growth of naturally occurring tumors. The data suggest that this mechanism of immune enhancement of naturally occurring tumors is likely to be operative against any tumor-specific transplantation antigen to which the host is chronically exposed. Since humans and most higher primates do not appear to have viral structural proteins present in their spontaneous tumors, it is felt that this line of experimentation should be continued after the transforming proteins are further characterized. (The genes encoding these transforming proteins will most probably be cloned from primate cells as they have from lower species). Some of these transforming proteins will most likely be transplantation antigens which should be examined immunologically and in immunobiology experimentation as has been outlind above for the viral structural proteins.

The viral vectors carrying the transforming genes have made it possible to isolate, mutagenize and insert these oncogenic gene sequences into different cells. In addition to genetic characterization this has provided the opportunity to define and characterize the transforming proteins. The production of a library of monoclonal antibodies reactive with single antigenic determinants of these proteins will make possible the search for these proteins in primates; the evaluation of these proteins as potential transplantation antigens; the fine structure analysis of these proteins which will lead to a better understanding of their mechanism of transformation and how to biochemically alter it; and finally, these reagents will provide indispensable tools to more rapid progress in one of the most promising areas in basic cancer research.

Proposed Course:

Immune cells from regressor, progressor and hyperimmunized rats will be examined by cell fusion and hybridoma production for production of monospecific antibodies. The immunogens of current interest include FeSV and AbLV transformed nonproducer cell lines. Immunoglobulin production from such hybrids will be examined for reactivity with viral and cellular encoded transforming proteins using immunoprecipitation and gel electrophoresis techniques.

Publications:

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Kende, M., Veronese, F., Hill, R., Stephenson, J.R., and Kelloff, G.J.: Natural killer cell activity and humoral blocking activity to endogenous type C viruses of the mouse: Interaction of these two immune responses in the aging mouse and its implications for natural tumorigenesis. Cancer Res., in press.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04942-11 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of cellular Genes Products with Transforming Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John R. Stephenson	Visiting Scientist	LVC	NCI
OTHER:	Gary J. Kelloff	Senior Surgeon	LVC	NCI
	Jonas Blomberg	Visiting Fellow	LVC	NCI
	Fulvia Veronese	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any)

F.H. Reynolds and W.J.M. Van de Ven, Litton Bionetics, Inc. (FCRC) Frederick, MD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	1.6	1.6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previously identified translational products of several acute RNA transforming viruses including Abelson murine leukemia virus and the Gardner and Snyder-Theilen strains of feline sarcoma virus (FeSV) have been found to possess tyrosine-specific protein kinase activity. Through the isolation of transformation-defective mutants, each of these viral gene products was shown to have transforming function. Specific tyrosine acceptor sites within both these viral transforming proteins and within a 150,000 M_r cellular substrate have been identified. Transformation by each of these viruses, as well as by McDonough FeSV, which does not encode a tyrosine protein kinase activity, was associated with reduced binding of epidermal growth factor (EGF). Transforming growth factors released by viral transformed cells and human tumor cells were found to stimulate tyrosine phosphorylation of the EGF membrane receptor. In other studies nucleic acid sequence homology was demonstrated between transforming genes of avian and mammalian cellular origin, establishing their highly conserved nature. Finally, in studies performed in collaboration with S. Oroszlan, proteoytic cleavage sites in polyproteins encoded by endogenous cat and baboon type C viruses were characterized.

Project Description

Objectives:

To determine the basic cellular regulatory pathways involved in malignant transformation. As one of several approaches to this general objective, type C viruses are being utilized as vectors for cloning cellular genes with transforming function. In addition, the involvement of proteins analogous to those encoded by type C transforming viruses, in spontaneous and chemically induced tumors of their natural hosts, is being explored. Immunologic reagents with specificity for transforming proteins developed through these studies are being utilized to explore cellular metabolic pathways involved in transformation of human cells.

Methods Employed:

Cell culture techniques including in vitrofocus assays, isolation of transformation-defective viral and cellular mutants, and development of hybridoma cell lines; biochemical and immunological procedures for protein analysis including gel electrophoresis, isoelectric focusing, immunoprecipitation, tryptic peptide mapping and phosphoamino acid determinations; molecular cloning of viral and cellular genes in prokaryotic vectors, application of expression plasmids to identification of gene products and use of nucleic acid sequencing techniques for analysis of cloned genes.

Major Findings:

1. Characterization of polyprotein translational products of the Gardner and Snyder-Theilen strains of feline sarcoma virus (FeSV). The Gardner and Snyder-Theilen strains of FeSV encode 115,000 molecular weight (M_r) polyproteins (P115's) as their primary translational products. Cells transformed by an independent variant of Snyder-Theilen FeSV, express an 85,000 M_r protein (P85) which corresponds to the amino terminal two-thirds of Snyder-Theilen FeSV P115. Single tyrosine phosphorylation acceptor sites have been identified within each of these viral gene products and are shown to be phosphorylated both in vivo and by the polyprotein associated protein kinase activities in immunoprecipitates. By two dimensional tryptic peptide analysis, we have shown that these acceptor sites, although highly related, do differ significantly. These differences are consistent with the possibility of one or more base substitutions subsequent to the recombinational event(s) leading to acquisition of cellular transforming genes by the feline leukemia virus genome. Finally, we have shown that each of these FeSV-encoded polyproteins are membrane components and that their tyrosine acceptor sites are phosphorylated both in purified membrane preparations and by incubation of intact cells in [^{32}P] ATP containing medium.

2. The high molecular weight polyprotein translational product of Snyder-Theilen FeSV has transforming function. Several transformation-defective (td) mutants of the Snyder-Theilen strain of FeSV have been isolated. Mink cells nonproductively infected by such mutants are morphologically nontransformed, fail to grow in soft agar, bind epidermal growth factors (EGF) as efficiently as control mink cells and lack rescuable transforming virus. Although levels

of expression of the major viral polyprotein translational product (P85) in td mutant infected clones are comparable to those of wild type (wt) transformants, polyproteins expressed in td mutant clones lack detectable protein kinase activity. Moreover total cellular phosphotyrosine levels are not elevated significantly above control values. Of a large number of wt Snyder-Theilen FeSV transformed mink cell clones isolated, the majority were found to revert to a nontransformed morphology upon continuous passage in cell culture. Such nontransformed variants, as well as a Gardner FeSV transformed mink cell revertant clone, lacked detectable polyprotein expression and exhibited levels of phosphotyrosine and EGF binding similar to those of control mink cells. These findings provide strong evidence favoring the involvement of the Gardner and Snyder-Theilen FeSV encoded polyproteins and their associated tyrosine-specific protein kinase activities in transformation.

3. Differences in mechanisms of transformation by independent FeSV isolates. In contrast to the Gardner and Snyder-Theilen strains of FeSV, McDonough FeSV encodes, as its major translational product, a 170,000 M_r polyprotein with probable transforming function, but lacking detectable protein kinase activity. Total cellular levels of phosphotyrosine remain unaltered in McDonough transformed cells and the major McDonough FeSV polyprotein translational product lacks binding affinity for a 150,000 M_r cellular phosphoprotein, P150, previously shown to specifically bind Gardner and Snyder-Theilen encoded gene products. These findings argue for major differences in the mechanisms of transformation by these independently-derived FeSV isolates.

4. Abelson murine leukemia virus transformation involves loss of epidermal growth factor binding sites. Cells transformed by the Abelson strain of murine leukemia virus (AbLV) were shown to resemble mammalian sarcoma virus transformed cells in that they also exhibited reduced levels of EGF binding. EGF binding was restored to control levels following loss of polyprotein expression in morphologic revertants of AbLV transformed clones. Similarly, EGF remained at high level in cell lines infected with AbLV td mutants previously isolated in our laboratory and shown to encode polyproteins deficient in tyrosine-specific protein kinase activity. These findings raise the possibility that the AbLV-polyprotein associated protein kinase activity mediates transformation through a mechanism directly or indirectly involving abolition of EGF binding sites.

5. Identification of tryptic peptides unique to a 110,000 molecular weight polyprotein encoded by the T-8 isolate of murine leukemia virus. Mink cells nonproductively infected with the weakly-transforming T-8 isolate of murine leukemia virus (MuLV) express a 110,000 M polyprotein designated T-8-MuLV P110. By immunoprecipitation analysis, T-8-MuLV P110 was shown to contain AKR-MuLV amino terminal gag gene-specific components (p15, p12) but to lack p30, p10, gp70, and p15(E) antigenic determinants. Of seven methionine-containing T-8-MuLV P110 tryptic peptides, at least four were unique and not represented in either AKR-MuLV $Pr180^{gag/pol}$ or $Pr82^{env}$. A clonal mink cell line nonproductively infected by the T-8 virus, and expressing high levels of P110, was shown to lack the elevated levels of tyrosine-specific protein kinase activity and reduction of EGF binding sites characteristic of many other viral transformed cell lines. These findings argue either that the T-8-MuLV genome contains acquired cellular sequences encoding a portion of

P110, or that T-8-MuLV P110 represents an inphase deletion of AKR-MuLV Pr180$^{gag/pol}$ with extensive post-translational modification and that an as yet unidentified protein is responsible for T-8-MuLV associated transformation.

6. Homology exists among the transforming sequences of avian and feline sarcoma viruses. The genomic RNA of the Fujinami strain of avian sarcoma virus (FSV) was shown to hybridize with RNA of PRCII sarcoma virus of chickens to a final extent of 56% and with RNAs of Gardner and Snyder-Theilen strains of FeSV to extents of 27% and 19%, respectively. In contrast, FSV cDNA lacked nucleotide sequences related to the src gene of Rous sarcoma virus, Y73 avian sarcoma virus, several representative avian acute leukemia viruses, AbLV or McDonough FeSV. Studies on thermal denaturation of hybrids showed that the melting temperatures of the heteroduplexes of the FSV cDNA with RNAs of PRCII and Gardner FeSV were 7°C and 12°C lower, respectively, compared with the melting temperature of the homologous hybrid of FSV, and suggested less than 10% mismatching in both heteroduplexes.

7. Human growth factors specifically induce phosphorylation of tyrosine residues in epidermal growth factor membrane receptors. Incubation of the A431 human tumor cell line in medium containing either EGF or human tumor cell-derived transforming growth factor (TGF) led to reduced EGF binding and a pronounced increase in overall cellular phosphotyrosine. Increased phosphotyrosine levels were also observed in response to a 20,000 M_r transforming growth factor (SGF) purified from the medium of Moloney-MSV transformed mouse cells. By use of in vitro labeling conditions favoring phosphorylation of tyrosine residues, a major 160,000 M_r substrate phosphorylated in response to binding of either human TGF or mouse sarcoma growth factor (SGF) was identified. By serologic analysis this substrate corresponded to the protein phosphorylated in response to EGF. The predominate phosphoamino acid, in each case, was identified as phosphotyrosine and three major P160 acceptor sites common to both EGF, SGF and TGF treated cells as well as several minor P160 peptides phosphorylated in response only to EGF, were identified.

8. Amino- and carboxyl-terminal sequences of proteins coded by gag gene of endogenous baboon and cat type C viruses. The amino- and carboxyl-terminal amino acid sequences of proteins (p10, p12, p15, and p30) encoded by the gag gene of endogenous baboon and cat type C viruses were determined. Among these proteins, p12s from both viruses had blocked amino termini. Proline was found to be the common amino end of both p30s and of cat p15, while alanine was the amino terminus of baboon p15 and both p10s. The amino-terminal sequences of the p30s differed at only one of the first 27 residues, while the p15s and p10s were distinctive but showed substantial homology. Leucine was found to be the carboxyl-terminal amino acid for p10, p12, and p30 from both viruses, while phenylalanine was the carboxyl-terminus of both p15s. Based on these amino acid sequences and the previously proposed NH_2-p12-p15-p30-p10-COOH polypeptide sequence in the precursor polyprotein, a model for proteolytic cleavage sites involved in the post-translational processing of the precursor coded for by the gag gene was proposed.

Significance to Biomedical Research and the Program of the Institute:

By the use of type C viruses as vectors for cloning genes with transforming function, we hope to identify and characterize transformation-specific proteins encoded by the cellular analogues of these viral genes and determine their possible role in spontaneous and chemically induced tumors of man.

Proposed Course:

In an effort to resolve whether cellular genes analogous to those sequences cloned in RNA transforming viruses are involved in spontaneous and chemically induced tumors of their natural hosts, several independent avenues of research will be pursued. As one approach, we will attempt to identify and characterize cellular analogues of the acquired sequence encoded components of FeSV and AbLV polyprotein translational products. In combination with conventional serologic studies, appropriate monoclonal immunologic reagents with specificity for viral and cellular encoded transforming proteins will be developed. Peptide mapping studies as well as a more detailed analysis of the enzymatic properties and substrate specificities of transformation-associated protein kinase activities will also be continued. In particular, amino acid residues phosphorylated by such kinases will be characterized and attempts made to identify their natural cellular substrates. In addition, molecular cloning techniques will be utilized for structural analysis of translational products of cellular genes analogous to those naturally cloned in type C viruses. Molecularly cloned viral and cellular transforming genes will be subcloned in expression plasmids as a means of identifying and characterizing their translational products.

Publications:

Stephenson, J.R., Van de Ven, W.J.M., Khan, A.S., and Reynolds, F.H. Jr.: Mammalian RNA type C transforming viruses: Characterization of virus-coded polyproteins containing phosphorylated components with possible transforming function. Cold Spring Harbor Symp. Quant. Biol. 44: 865-874, 1980.

MacDonald, M.E., Reynolds, F.H., Jr., Van de Ven, W.J.M., Stephenson, J.R., Mak, T.W., and Bernstein, A.: Anemia- and polycythemia-inducing isolates of Friend spleen focus-forming virus: Biological and molecular evidence for two distinct viral genomes. J. Exptl. Med. 151: 1477-1492, 1980.

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Shibuya, M., Hanafusa, T., Hanafusa, H., and Stephenson, J.R.: Homology exists among the transforming sequences of avian and feline sarcoma viruses. Proc. Natl. Acad. Sci. USA 77: 6536-6540, 1980.

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Jakowski, R.M., Essex, M., Hardy, W.D. Jr., Stephenson, J.R., and Cotter, S.M.: Membranous glomerulonephritis in a household cluster of cats persistently viremic with feline leukemia virus. In Hardy, W.D. Jr., Essex, M., and McClelland, A.G. (Eds.): Feline Leukemia Virus. Amsterdam, Elsevier/North Holland Biomedical Press, 1980, pp. 141-149.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05070-03 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Temperature Sensitive Mutants of Moloney Murine Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Donald G. Blair	Expert	LVC	NCI
OTHERS:	Peter J. Fischinger	Medical Director	LVC	NCI
	William G. Robey	Research Chemist (Biochem.)	LVC	NCI
	Thomas G. Wood	Senior Staff Fellow	LMV	NCI

COOPERATING UNITS (if any)

R.B. Arlinghaus, Dept. of Tumor Virology, M.D. Anderson Hospital and Tumor Institute, Houston, TX

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Virus Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.3	OTHER: 0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genetic and biochemical studies on ts110, a Moloney murine sarcoma virus (MSV) temperature-sensitive (ts) mutant which transforms cells at 34 but not at 39 , have been extended. At least 0.3 X 10^6 daltons of genetic information has been deleted from ts110 MSV, when compared to its wild type (wt) parent. Furthermore, ts110-transformed normal rat kidney cells contain an additional major RNA species approximately 0.6 X 10^6 daltons smaller then the single major species found in wt-transformed cells.

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Project Description

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses (MSV) and the function and cellular targets of specific gene products of MSV in this process.

Methods Employed:

Focus and agar colony formation by MSV on various cell lines in the presence and absence of replicating helper viruses; DNA transfection using cloned MSV sequences and whole cell DNA-transformed cells; recombinant DNA cloning of integrated MSV sequences from transformed cells; restriction endonuclease mapping and heteroduplex analysis of cloned sequences.

Major Findings:

Mutant virus shows an altered intracellular viral RNA pattern. Normal rat kidney (NRK) cells transformed by MSV temperature sensitive (ts)110 contain two size classes of *mos* containing polyadenylated RNA, while cells transformed by the wild type (wt) parent of ts110, MSV 349, contain only a single detectable RNA species. The two species present in ts110 are approximately 0.3 X 10^6 and 0.6 X 10^6 daltons smaller respectively than the single, approximately 2.2 X 10^6 dalton wt MSV 349 RNA. Both species are also present in a spontaneous wt revertant of ts110. Both species are present in ts110 cells grown at either the permissive (34°) or nonpermissive (39°) temperature, although in some experiments the relative amount of the smaller species is reduced. The results suggest ts110 virus has undergone a deletion, and is altered in its method of RNA expression or processing, but whether either defect is related to the ts transformation phenotype is unclear.

Significance to Biomedical Research and the Program of the Institute:

Conditional mutants have been extremely useful in elucidating biochemical pathways in a number of cell and viral systems. The MSV mutant ts110 represents one of the few stable conditionally defective variants of a mouse-derived sarcoma virus. Further analysis of these and similar mutants should help to elucidate the mechanism of transformation by Moloney MSV.

Proposed Course:

A restriction map of ts110 MSV provirus will be obtained and compared to the wt MSV 349 to attempt to determine the nature and location of the deletion or deletions present in the genome. Further attempts will be made to clone the ts genome either as an integrated provirus or directly from circular genomes isolated from the Hirt supernatant. These clones will be used to directly define the site of the ts lesion and to map the structure of the two RNA species by r-loop electron microscopy and Burke and Sharp electrophoretic analysis. Attempts will be made to complement the ts genome which was transferred to NIH 3T3 cells by DNA transfection.

Publications:

Wood, T.G., Horn, J.P., Robey, W.G., Blair, D.G., and Arlinghaus, R.B.: Characterization of viral specified proteins present in NRK cells infected with a temperature-sensitive transformation mutant of Moloney murine sarcoma virus. Cold Spring Harbor Symp. Quant. Biol. 44: 747-754, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05071-03 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Experimental Basis for a Molecular Approach to Cancer Prophylaxis and Therapy for Some Types of Human Neoplasia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leo A. Phillips	Research Microbiologist	LVC	NCI
OTHER:	Mohinder S. Kang	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.8	PROFESSIONAL: 1.0	OTHER: 0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to make possible a molecular approach to cancer prophylaxis and therapy for some types of viral neoplasia, especially human leukemia, cancer of the breast, and other human neoplasia in which retrovirus information may be detected. Major findings to date are: (1) adenosine (A), cytidine (C), and guanosine (G) tracts are present in all retrovirus RNAs examined; (2) uridine (U) tracts are absent in retrovirus RNA; (3) C and G tracts present in retrovirus RNA are transcribed into complementary deoxyguanosine (dG) and deoxycytidine (dC) tracts, respectively, in cDNA transcripts of retrovirus RNA; (4) the 3'-terminal poly(A) sequences are not transcribed into poly (dT) sequences; (5) no sequences of like nucleotides of $\geq$ 10 bases in length are present in the DNA of nonmalignant human embryonic cells; (6) however, dCMP and dGMP tracts of $\geq$ 5 bases in length are present in the DNA of spontaneously transformed and retrovirus infected human embryonic cells and in the DNA of retrovirus infected and transformed, as well as chemically transformed mammalian cells; (7) DNA from a variety of excised human tumors and from various human tumor cell cultures are to be examined for the presence or absence of tracts of dCMP and dGMP nucleotides.

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Project Description

Objectives:

The objective of this project is to establish a sound scientific basis for a molecular approach to cancer prophylaxis and therapy for human neoplasia in which retrovirus information may be detected. Studies are divided into four phases. (I) The biochemistry and biophysics of retrovirus and cellular nucleic acids: To look for and to characterize, when detected, unique specific nucleotide sequences present in retrovirus nucleic acids which may either be absent or present in insignificant amounts in cellular nucleic acids from malignant or nonmalignant mammalian and human cells. (II) The in vitro testing of synthetic single-stranded polymers, chemically modified antibiotics, sequence specific drugs and monoclonal antibodies to restriction endonuclease generated cDNA fragments containing tracts of deoxycytidine 5'-monophosphate (dCMP) and deoxyguanosine 5'-monophosphate (dGMP) nucleotides or other viral-specific nucleotides detected from sequencing data to determine whether these agents can specifically inhibit transcription of integrated retrovirus proviral genes without interfering significantly with the transcription of cellular genes. (III) The in vivo testing of synthetic single-stranded polymers, chemically modified antibiotics, sequence-specific drugs and monoclonal antibodies to restriction endonuclease generated cDNA fragments containing deoxycytidine (dC) and deoxyguanosine (dG) tracts or other viral specific nucleotides detected by sequencing data to determine whether the transcription of integrated retrovirus proviral genes can be selectively blocked in mammals that have a high incidence of breast cancer and leukemia by *in situ* hybridization of nuclease protected, single-stranded polymers complementary to dC and dG tracts present in integrated retrovirus proviral DNA and the specific binding or intercalation of sequence-specific drugs and chemically modified antibiotics to these like sequences of dCMP and dGMP in the viral DNA. Sufficient testing will be done to acquire investigatory new drug (IND) numbers from the Federal Drug Administration (FDA). (IV) The prophylactic and therapeutic use of the potential anti-cancer agent(s) found to be most effective in the phase III animal studies in humans. During the period of this report, work has emphasized primarily Phase I of this program. Work on Phase II has begun. No work on Phases III and IV was started.

Methods Employed:

Analytical and preparative ultracentrifugation; RNA-DNA and DNA-DNA hybridizations; DNA isolation, molecular cloning and sequencing; reverse transcription, nick translation, Southern transfer, and restriction endonuclease mapping; viral and messenger RNA isolation and fractionation, affinity and column chromatography, Northern transfer, rate zonal and density gradient centrifugation; gel electrophoresis, agarose and polyacrylamide gels, autoradiography, and gel scanning; isotopic labeling with [^{3}H], [^{14}C], [^{32}P], [^{125}I] radionuclides, peptide and oligonucleotide fingerprinting; radioimmunoassays, immunodiffusion, immunoprecipitation, monoclonal antibody production; amino acid analysis by high performance liquid chromatography (HPLC). Tissue culture techniques for initiation, growth, maintenance, passage and synchronization of mammalian and human cell cultures, for retrovirus production, for infectivity assays of retroviruses, and for in vitro testing of anti-cancer agents.

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Major Findings:

1. Elucidation of the structure, size, and nucleotide sequence composition of retrovirus RNA. The genomic RNA of Gazdar murine sarcoma virus (Gz-MSV/MuLV) is composed of 28S to 30S, or 5.0 to 5.8 kilobase (kb) length, subunit RNA and 34S to 35S, or 7.5 to 7.8 kb length, subunit RNA molecules. The 5.0 to 5.8 kb RNA contains the sarcomagenic information; whereas, the 7.5 to 7.8 kb RNA contains the leukemogenic information. The genomic RNA of Gz-MSV/MuLV and Rauscher murine leukemia virus (R-MuLV) contain segments of double-stranded RNA and there appears to be both interstrand double-stranded regions between the subunit RNA forming the genomic RNA and intrastrand double-stranded regions within the subunit RNA. Most of the subunit RNA of Gz-MSV/MuLV, but not all, has 3'-terminal poly(A) sequences, or tracts rich in adenosine 5'-monophosphate (AM)) of variable chain lengths of up to 0.3 kilobases (kbs). Retrovirus RNA from avian to primate isolates also contain internal tracts of 5 nucleotides of cytidine 5'-monophosphate (CMP) and guanosine 5'-monophosphate (GMP). However, tracts ≧ 10 nucleotides of uridine 5'-monophosphate (UMP) are absent in retrovirus RNA. Enzymatically excised cytidine (C) tracts are approximately 70% CMP with ≧ 5 nucleotides of CMP, whereas enzymatically excised guanosine (G) tracts are more difficult, if not impossible, to characterize definitively as to their chain lengths because of their notorious ability to aggregate.

2. Transcription in vitro of retrovirus C and G tracts. The C and G tracts present in retrovirus RNA are transcribed in vitro to deoxyguanosine (dG) tracts of ≧ 5 nucleotides of deoxycytidine 5-monophosphate (dCMP) and to deoxycytidine (dC) tracts of ≧ 5 nucleotides of deoxycytidine 5-monophosphate (dCMP), respectively. The 3'-poly(A) sequences of retrovirus RNA are not transcribed into poly (dT) sequences in the complementary DNA (cDNA) transcripts of retrovirus RNA. Viral cDNA transcripts were synthesized in vitro in endogenous and exogenous reverse transcriptase reactions. Oligodeoxynucleotides of dC, dG, dT, and calf thymus DNA can prime the synthesis of cDNA from retrovirus RNA in the exogenous reverse transcriptase reaction and that the order of priming efficiency for these oligodeoxynucleotides are: oligo calf thymus DNA > oligo (dT) > oligo (dG) > oligo (dC) > oligo (dA) endogenous transfer RNA primer. Complementary binding of oligo calf thymus DNA primer to the template viral RNA does not appear to be a requirement for its priming activity. In contrast, the priming activity of oligo dC, dG, and dT does appear to require complementary binding to the viral RNA template.

3. Elucidation of the structure, size, and sequence of retroviral DNA. Six to 8.2 kilobase (kb) length cDNA transcripts were synthesized in the endogenous reverse transcriptase reaction. However, thus far we have only been able to synthesize 0.28 to 0.37 kb length DNA transcripts in the exogenous reverse transcriptase reaction. Our results indicate that dC tracts 5 nucleotides of dCMP and dG tracts ≧ 5 nucleotides of dGMP are present in cDNA transcripts of retrovirus RNA. However, dA tracts 10 ≧ nucleotides of dAMP and dT tracts 10 nucleotides of deoxythymidine 5'-monophosphate (dTMP) are absent in retrovirus cDNA. Several laboratories have now confirmed, by nucleotide sequencing, that the 3'-terminal poly(A) sequences are not transcribed because the 145 base strong-stop-DNA of the 5'-long terminal repeat (5'-LTR) binds to complementary bases in the 3'-LTR of a second subunit RNA molecule which is

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then transcribed in a 3' to 5' direction up to the 18 nucleotide binding site of the transfer RNA (tRNA) primer within the 5'-LTR of the second subunit RNA molecule.

4. Signal nucleotide tracts are present in transformed, but absent from normal human cells. These studies were performed to obtain evidence for the presence or absence of tracts of like nucleotides of dAMP, dCMP, dGMP, and dTMP in human and other mammalian cellular DNA. The existence of tracts of like nucleotides in retrovirus DNA and not in human cellular DNA would enhance the future possibility for the selective blocking of transcription of integrated retrovirus genes without interfering significantly, if at all, with the transcription of cellular genes. No tracts of like nucleotides ≧ 10 bases in length were detected in the DNA of nonmalignant human embryonic cells. However, tracts of ≧ 5 nucleotides of dCMP and dGMP were present in the DNA of spontaneously transformed and retrovirus infected human embryonic cells and in the DNA of virus infected and transformed, as well as chemically transformed, mammalian cells. We now wish to examine the DNA from a variety of human tumor biopsies, as well as human tumor cell cultures, for the presence or absence of dC and dG tracts.

Our Phase II studies on the in vitro testing of synthetic single-stranded polymers, chemically modified antibiotics, sequence-specific drugs and monoclonal antibodies to restriction endonuclease generated cDNA fragments containing tracts of dCMP and dGMP nucleotides to determine whether these agents can specifically inhibit transcription of integrated retrovirus proviral genes without interfering significantly, if at all, with the transcription of cellular genes have only recently been initiated after our Phase I studies suggested the possibility of a molecular approach to cancer. Thus far, we have only become proficient in biological assays for infectious and transforming retroviruses and in RNA-DNA and DNA-DNA hybridization techniques for the detection of viral DNA in virus-infected and transformed cells.

Significance to Biomedical Research and the Program of the Institute:

A molecular approach to cancer prevention and treatment for human cancers in which retrovirus information has been detected, primarily breast cancer and leukemia, has been justified, in part, from our biochemical and biophysical studies on the structure and nucleotide composition of retrovirus and cellular nucleic acids. Analysis of homoribonucleotide sequences in retrovirus RNA revealed the existence of 3'-terminal poly(A) sequences and internal C and G tracts. These sequences, or tracts, have been detected in the RNA of retroviruses from avian to primate isolates. Poly(A) sequences have also been found in all cellular messenger RNA (mRNA), except for histone mRNA, by many investigators. We have shown that comparatively little or no C and G tracts exist in poly(A)-positive mRNA examined from nonmalignant and embryonic cells. C and G tracts present in the viral RNA are transcribed into dG and dC tracts, respectively, in the viral cDNA, before it becomes integrated into cellular DNA where it apparently remains in an inactive, repressed state until it is activated or derepressed either spontaneously or by chemical or physical carcinogens. The absence of significant amounts of these dC and dG tracts in the cellular DNA from nonmalignant human embryonic cells and their presence in the DNA from retrovirus infected and transformed cells, as well as chemically

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transformed cells, suggest an experimental basis for a possible molecular approach to cancer prevention and treatment for cancer in which retrovirus information has been detected. Perhaps the transcription of the integrated viral genes with their unique sequences of dCMP and dGMP or other viral specific nucleotides detected from sequencing data could be selectively blocked without interfering with the transcription of cellular genes. Although the causes of human cancers in which retrovirus information has been detected are most probably multifactorial, this unique approach seems worthy of exploring for the prevention, primarily, and the treatment, secondarily, of cancer.

Proposed Course:

Phase I: These essential biochemical and biophysical studies on the nucleic acids of mammalian retroviruses and mammalian and human cells will be continued in concert with our Phase II studies. Our major research efforts will continue to be directed toward the establishment of a sound scientific basis for a molecular approach to cancer prevention and treatment. The following biochemical and biophysical studies on the nucleic acids of retroviruses and cells will be performed.

Planned RNA Studies: (1) Determination of the location(s) of the C and G tracts in the retrovirus RNA by controlled polynucleotide phosphorylase (PNPase) phosphorolysis of poly(A)-positive viral RNA subunits from their 3'-termini, by heteroduplex analysis when it becomes sensitive enough to resolve structures with less than 40 base pairs; (2) Demonstration of the base sequences of the C and G tracts in the viral RNA by two-dimensional chromatography in agarose of RNase T1 and RNase C excised tracts. Presently, the like sequences of CMP and GMP in retrovirus RNA have been detected by polynucleotide agarose affinity chromatography, by hybridization or annealing studies, by specific chemical binding studies, and by the ability of oligo(dC), and oligo(dG) to serve as primers in the in vitro synthesis of viral cDNA; (3) Determination of the base composition of excised double-stranded RNA (dsRNA) fragments from retrovirus RNA; (4) Determination as to whether the p12 viral polypeptide binds to the single-stranded or the double-stranded portion of genomic and subunit viral RNA; (5) Characterization of the interstrand and intrastrand double-stranded regions in the genomic and subunit RNA of mammalian retroviruses as to their nucleotide composition, their definitive chain-lengths, and their possible functions; (6) Determination of the rôle(s) of the poly(A) and C and G tracts in genomic and subunit viral RNA; (7) Continued examination of the poly(A) positive mRNA from nonmalignant cells, from retrovirus infected and transformed cells, and chemically transformed cells of human origin for the absence or presence of C and G tracts.

Planned DNA Studies: (1) Location of the dC and dG tracts in subunit-length cDNA copies of retrovirus RNA; (2) Continued examination of the DNA from nonmalignant cells, from retrovirus infected and transformed cells, from chemically transformed cells of human origin and from human tumors for the absence or presence in the dC and dG tracts; (3) Determination of the kilobase length of the cDNA transcripts when oligo(dC), oligo(dG) and oligo(dT) are used as primers in the in vitro synthesis of cDNA.

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Our Phase III studies on the in vivo testing of synthetic single-stranded polymers, chemically modified antibiotics, sequence specific drugs and monoclonal antibodies to restriction endonuclease generated cDNA fragments containing dC and dG tracts to determine whether the transcription of integrated retrovirus proviral genes can be selectively blocked in mammals are only in the planning stage and have not been initiated.

Phase IV: Our Phase IV studies on the prophylaxis and therapy of human neoplasia in which retrovirus information has been detected with the potential anti-cancer agents found to be most effective in our planned Phase III animal studies have not yet been initiated in cooperative studies with NCI practicing physicians, because the animal testing required to obtain an investigatory new drug number (IND Number) from the Food and Drug Administration (FDA), although planned, has not been initiated.

Publications:

Elbein, A.D., Heifetz, A., Kang, M.S., Spencer, J.P., and Keenan, R.W.: Inhibition of lipid-linked saccharide formation by antibiotics. In Marshall, J.J. (Ed.): Mechanisms of saccharide polymerization and depolymerization. New York, Academic Press, 1980, pp. 1-20.

Kang, M.S., Park, J.J., Singh, I., and Phillips, L.A.: Streptovirudin inhibits glycosylation and multiplication of vesicular stomatitis virus. Biochem. Biophys. Res. Comm. 99: 422-428, 1981.

Phillips, L.A., Kang, M.S., and Hollis, V.W. Jr.: Complementary DNA copies of leukemia and sarcoma virus RNA contain sequences of deoxycytidylate and deoxyguanylate. In Nieburgs, H.E. (Ed.): Cancer Detection and Prevention. New York, Marcel Dekker, Inc., in press.

Phillips, L.A., and Kang, M.S.: Structural components of retroviruses: viral RNA and DNA. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker, Inc., in press.

Phillips, L.A., and Kang, M.S.: Retroviruses: Experimental basis for a molecular approach to human cancer. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker, Inc., in press.

Aulakh, G.S., Aulakh, H.S., Hollis, V.W., Khan, A., and Phillips, L.A.: Implication of murine retrovirus related genetic information in human tumors. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker, Inc., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05078-03 LVC

PERIOD COVERED
October 1, 1980 to April 2, 1981

TITLE OF PROJECT (80 characters or less)

Immunological and Virological Studies in Epstein-Barr Virus Induced Malignancies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kamaraju Sundar	Visiting Fellow	LVC	NCI
Other:	Paul Levine	Medical Director	LVC	NCI
	Dharam Ablashi	Microbiologist	LCMB	NCI
	Kamaraju Sreemahalakshmi	Visiting Fellow	LVC	NCI
	Alberto Faggioni	Visiting Fellow	LCMB	NCI
	Sergio Leiseca	Research Biologist	LVC	NCI

COOPERATING UNITS (if any) J.F. Hewetson, J.L. Cicmanec, Litton Bionetics, Inc. (FCRC), Frederick, MD; L. Wolfe, St. Luke's Medical Center, Chicago, IL.; and M. Nonoyama, Life Sciences, St. Petersburg, FL

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Clinical Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.55	1.30	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immunologic techniques were applied to the study of Epstein-Barr virus (EBV) oncogenesis in nonhuman primates. Tumors and fatal lymphoproliferative disorders were produced in a white-lipped marmoset, common marmoset and owl monkey. Specific cell-mediated immunity, immunosuppression, and serum blocking factor were associated with tumor appearance in the white-lipped marmoset. A rabbit animal model was developed for Herpesvirus saimiri oncogenesis. Several anti-inflammatory agents and retinoic acid inhibited the production of EBV nuclear antigen, EBV antigens induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA), DNA synthesis and transformation.

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Project Description

Objectives:

To produce Epstein-Barr virus (EBV)-induced tumors in non-human primates as a model for EBV-associated tumors in man, with particular emphasis on the immune response. To study the mechanism of EBV replication and transformation using tumor promoters, anti-inflammatory agents and retinoids.

Methods Employed:

B95-8 Epstein-Barr virus (EBV) was inoculated into the bone marrow (BM) and peritoneum (IP) of six white-lipped marmosets (WLM) and owl monkeys. Heparinized blood was collected at 14 day intervals for serological and cellular immunity assays. Common marmosets were inoculated into BM and IP with EBV and TPA. Inbred strain III/J rabbits were inoculated with Herpesvirus saimiri (HVS) intravenously. Serum blocking factor was detected by the inhibition of specific cell-mediated immunity (CMI) response in the lymphocyte stimulation assay. Based on our results with serum blocking factor in experimental malignancy, we tested the sera of nasopharyngeal carcinoma (NPC) patients for serum blocking factors. The effect of the tumor promoting agent 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on the in vitro antigenic expression of oncogenic herpesviruses was studied by the immunofluorescence test and by the titration of infectivity. Similarly, the effect of TPA on EBV transformation was evaluated. In order to understand the mechanism of TPA induced antigenic expression, steroids were used in an attempt to inhibit EBV induced EBNA, DNA synthesis and transformation.

Major Findings:

1. Immunologic studies of EBV-associated tumors in primates. One of six WLM developed diffuse malignant lymphoma 55 after days after inoculation with the transforming B95-8 strain of EBV, and a fatal lymphoproliferative disorder developed in an inoculated owl monkey. Autopsy of the WLM revealed enlarged maxillary, mandibular, cervical and mesenteric lymph nodes. Histopathology of these lymph nodes showed well differentiated lymphocytes with an overlying area of inflammation and necrosis. The normal architecture of the lymph nodes was replaced by the infiltrating lymphocytes which included numerous cells with mitotic figures and degenerating nuclei. EBV DNA was detected in one of the enlarged lymph nodes. The affected animal had high antibody titers to the viral capsid antigen (VCA) and early antigen (EA). The animal developing diffuse malignant lymphoma had a significant CMI response to EBV antigens and to phytohemagglutinin (PHA), which became depressed as the tumor progressed. Serum obtained from the affected animal during this period had a blocking factor which decreased or blocked the response of mononuclear cells from clinically normal animals to PHA. In the owl monkey, EBV genome was detected in the abnormal and in the enlarged lymph nodes.

Similar immunologic studies were performed in patients with NPC; a serum blocking factor which abrogated the response of normal mononuclear cells to

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EBV antigens was detected in all 25 patients studied. In 9 cases, serum blocking factor disappeared with successful therapy as measured by declining tumor burden while high levels persisted in non-responsive cases. None of the sera collected from 15 patients with other head and neck cancer or 10 healthy individuals had serum blocking factor.

2. Studies on the mechanism of EBV replication and transformation. TPA induced and/or enhanced the antigen expression of oncogenic herpes viruses in vitro and enhanced the transformation of mononuclear cells by EBV. The anti-inflammatory agents inhibited the TPA-induced antigenic expression in Raji cells. The steroids and retinoic acid inhibited EBV-induced EBNA, cellular DNA synthesis and transformation.

Significance to Biomedical Research and the Program of the Institute:

EBV has been implicated as causing a variety of diseases in humans, ranging from inapparent infection to lymphoma. The development of an animal model for EBV infection is important to studies on the etiology, host-virus interactions and control of EBV associated diseases. Although cotton-topped marmosets (CTM) are the most susceptible for EBV induced lymphomas, an alternate animal model is necessary because CTM are an endangered species available in limited numbers for biomedical research. WLM are available in considerable numbers. This study not only suggests WLM as an alternate animal model for EBV infection and cancer but also demonstrates the feasibility of investigating cell mediated immune response in these animals. This animal model will be useful in further investigating the pathogenesis of EBV, the effect of co-carcinogens, and the effectiveness of antiviral agents in the control of EBV-induced malignancy. The demonstration that rabbits are susceptible to oncogenic herpes viruses provides additional cost-effective animal models for EBV-induced lymphoma. The test for serum blocking factor may be useful to monitor the response of NPC patients to therapy and may also be helpful in detecting relapse in an early stage. The in vitro experiments with tumor promoters and steroids are helpful to understand the role of cocarcinogens in EBV oncogenesis and for understanding the mechanisms involved.

Proposed Course:

This project has terminated and other section investigators will continue portions of these projects.

Publications:

Sundar, K.S., Ablashi, D.V., Bengali, Z.H., Levine, P.H., and Nonoyama, M.: Mitogenic effect of 12-O-tetradecanoyl-phorbol-13-acetate on non-human primate mononuclear cells and in vitro interaction with EBV transformation. Arch. of Virol. 64: 141-153, 1980.

Ablashi, D.V., Bengali, Z.H., Eichelberger, M.A., Sundar, K.S., and Levine, P.H.: Increased infectivity of primate oncogenic herpesviruses with tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate. Proc. Soc. Exp. Biol. Med. 164: 485-490, 1980.

Ablashi, D.V., Sundar, S.K., Armstrong, G.R., Galoway, P., Valerio, M., Bengali, Z.H., Kemp, J., and Fox, R.R.: *Herpesvirus saimiri* induced malignant lymphoma in inbred strain III/J rabbits (*Oryctolagus cuniculus*). J. Cancer Res. and Clin. Oncol. 98: 165-172, 1980.

Sundar, S.K., Levine, P.H., Ablashi, D.V., Leiseca, S.A., Armstrong, G.R., Cicmanec, J.L., Parker, G.A., and Nonoyama, M.: Epstein-Barr virus induced malignant lymphoma in a white-lipped marmoset. Int. J. Cancer 27: 107-111, 1981.

Sundar, S.K., Ablashi, D.V., Levine, P.H., Wallen, W.C., and Armstrong, G.R.: Tumor promoting agent induces lymphocyte mitogenic factor. Biomed., in press.

Sundar, S.K., Lai, P.K., Bengali, Z.H., Cicmanec, J.L., and Goldstein, K., Levine, P.H.: *In vitro* blastogenic responses of marmoset peripheral blood lymphocytes to phytohemagglutinin and to autologous lymphocytes transformed by Epstein-Barr virus. Comp. Immunol. Microbiol. Inf. Dis., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05099-03 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure, Organization and Biological Activity of Primate Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Narayana Battula	Visiting Associate	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.15	PROFESSIONAL: 0.85	OTHER: 0.30

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Baboon endogenous type C leukemia viral DNA intermediates have been purified from cells acutely infected with the virus. The circular viral DNAs were linearized by digestion with the restriction endonuclease EcoRl and the linearized DNA was molecularly cloned in bacteriophage λ by recombinant DNA techniques. To prepare specific probes for subgenomic fragments of viral DNA, the full length cloned DNA was restricted into 5 fragments and each of the fragments were independently cloned in the plasmid PBR 322. The organization of the endogenous genetically transmitted viral sequence in baboon DNA and the exogenous horizontally transmitted sequence acquired upon horizontal infection of human and dog cells were compared by Southern blotting procedure. The genetically transmitted sequences and the sequences acquired upon infection showed organizations similar to the DNA of the infectious virus. However, the DNA from virus infected cells (horizontally transmitted sequences) are infectious in DNA transfection assays but the DNA from baboons (genetically transmitted sequences) are 2 to 3 logs less infectious. The factors that potentially regulate viral gene expression are under investigation.

Project Description

Objectives:

To characterize the distribution within primates of nucleic acid sequences related to the baboon endogenous type C virus, its sequence organization and the factors influencing its biological activity.

Methods Employed:

To prepare the different molecular forms of viral DNAs a variety of hydrodynamic, chromatographic and electrophoretic techniques were employed. To assay for the biological activity of the integrated and unintegrated viral DNAs transfection assays were carried out using human or dog cell recipients. To molecularly clone viral DNA by recombinant DNA techniques, bacteriophage vectors, in vitro packaging of λ, and colony-blot methods were used. To subclone total viral DNA and its subgenomic fragments the plasmid pBR 322 vector was used. To detect viral specific sequences labeled probes were made using calf thymus DNA primers or by nick translation. The DNA was transferred onto nitro cellulose papers by the Southern procedure and hybridized with labeled probes.

Major Findings:

1. Covalently closed circular DNAs of the baboon endogenous type C virus were purified. Analysis of the circular DNAs showed that they consist of two molecular forms. The larger form was about 9.0 Kilobases (kb) and contained two terminal repeat sequences and the smaller was about 8.4 kb and contained a single terminal repeat. They differed from each other by a single 0.6 kb terminal repeat sequence.

2. The circular viral DNAs were molecularly cloned in bacteriophage by recombinant DNA techniques. The circular DNAs were linearized by digestion with the restriction endonuclease EcoR1 which has a unique recognition site on the viral DNA. The linearized, circularly permuted DNA was ligated to the EcoR1 arms of λ WES. λ B vector and the ligated molecules were then packaged in vitro into infectious particles and amplified by lytic growth on EK2 host. The plaques were screened for M7 specific sequences and the recombinants selected. The DNAs from four recombinants were purified and analyzed. Two of the four recombinants contained a single terminal repeat and the other contained two terminal repeat sequences.

3. Cloned full length M7 DNA and its subgenomic fragments were subcloned in PBR 322. Cloned M7 DNA was digested with Bam H1 and the resulting five fragments were purified. Each of the five fragments and the full length DNA were independently ligated at their appropriate restriction sites to the plasmid PBR 322, propagated and the plasmids containing the inserts were characterized.

4. The organization of the endogenous and exogenous sequences was investigated using viral specific probes. Baboon cells contain approximately 100 copies of genetically transmitted M7 sequences (endogenous sequences) and the

virus infected human and dog cell clones contain less than 10 copies (exogenous sequences) per cell. The organization of the multiple sequences was surprisingly simple and there were no substantial deletions or substitutions in the viral DNA. The endogenous sequences have the same size and arrangement similar to the unintegrated viral DNA and therefore, approximately similar to the RNA of the infectious virus. The endogenous sequences are colinear with the DNA of the infectious virus and are integrated at different sites in the baboon DNA much after the manner of the M7 proviral DNA sequences acquired upon horizontal infection.

Significance to Biomedical Research and the Program of the Institute:

The association of retroviruses with spontaneous or natural leukemias has been documented in chickens, mice, cats, cows and monkeys. This information led to the search for the presence of retroviruses in higher primates including man. A number of retroviruses have since been isolated from primates and not from man. Baboons, evolutionarily, are man's closest relatives from which a retrovirus has been isolated. Therefore, we used cloned viral DNA of baboon retrovirus to identify related sequences in humans. We have not been successful in detecting M7 virus-related sequences in humans. We are currently trying to define fragments of viral genomes that are specifically conserved which might allow detection of related human sequences and determine if these are altered in disease.

Proposed Course:

The arrangement of both the genetically transmitted sequences and those acquired upon horizontal infection are apparently similar. The infected cells produce high titer virus and their DNA is infectious. The baboon tissues or cells, however, do not readily produce virus and the infectivity of their DNA is two to three logs lower than that of the infected cells. These observations indicate that, in addition to sequence arrangements, other factors such as DNA modifications by methylation influence viral gene expression. We will investigate some of the factors that potentially regulate viral gene expression by comparing the genetically transmitted endogenous viral sequences with the sequences acquired upon horizontal infection.

Publications:

Battula N., and G.J. Todaro: Physical map of infectious baboon type C viral DNA and sites of integration in infected cells. J. Virol 36: 709-718, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05108-03 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Growth Factors Released by Transformed Cells: A Possible Mechanism Responsible for the Transformed Phenotype

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joseph E. De Larco	Research Chemist	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	Hans Marquardt	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	0.95	0.30

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sarcoma growth factors (SGFs) are peptides capable of interacting with the epidermal growth factor (EGF) receptor system and phenotypically transforming non-transformed cells in tissue culture. These peptides are isolated from serum-free conditioned media of murine sarcoma virus (MSV) transformed cells. This conditioned media contains several peptide factors capable of modifying the growth or differentiation patterns of biological systems. A SGF with an apparent molecular weight of 10,000 has previously been characterized. This is shown not to be a direct product of the sarcoma genome; its expression, however, is controlled by the product of the sarcoma genome. Recently a 20,000 molecular weight peptide has been characterized and purified to apparent homogeneity using exchange and high pressure liquid chromatographies. This peptide competes on a mole for mole basis with EGF for its receptor and is capable of stimulating its phosphorylation. This peptide will be sequenced sufficient quantities have been purified.

Project Description

Objectives:

To purify and sequence the major classes of growth factors released by transformed cells that interact with untransformed fibroblastic cells to allow them to grow in an anchorage independent manner. When enough purified materials have been accumulated the amino acid sequences will be determined and these will be compared with each other as well as to the sequences of characterized peptide hormones and growth factors to determine if any homologies exist.

Once the amino acid sequences have been determined nucleotide probes can be constructed to purify the messenger RNAs (mRNAs) for these factors. Reverse transcribed complementary DNAs (cDNAs) from the mRNAs will allow the cloning of these genes. With clones available the transcription and expression of these genes can be monitored during development as well as in neoplasia. Along parallel lines the purified growth factors or synthetic peptides corresponding to known sequences found in the sequenced factors will be used to develop antibodies for radioimmune assays. These assays will be used to determine if these factors or peptides immunologically related to them are expressed either during development or by certain tissues under normal physiological conditions. The level of expression in tumors will be examined and compared with those of homologous normal tissues.

The interaction of these growth factors with normal cells and their subsequent biological modification will be studied to determine the sequence of events required for phenotypic transformation and contrasted with events that occur when known growth factors are used (i.e. the interaction of epidermal growth factor (EGF) with its receptor that stimulates the phosphorylation of the EGF receptor).

Transformed cells grown in vitro show a multiplicity of changes when compared to their untransformed counterparts. Transformed cells in tissue culture express a morphological change as seen in their growth pattern; while normal cells tend to grow in flat, regular geometric patterns, transformants tend to criss-cross over one another and form multiple layers containing cells of heterogeneous size and shape. Other major alterations of transformed cells are found in their growth characteristics. Nontransformed fibroblastic cells require attachment to a substrate for proliferation, a property referred to as "anchorage dependent growth". The transformed cell has a decreased adhesion to its substrate and may have acquired the ability to grow in an anchorage independent manner. Growth in semisolid media or suspension is an indication of anchorage independent growth and is considered a good criterion of transformation. Proliferation of untransformed cells in tissue culture requires growth factors that are either supplied by serum or added as purified components to defined media. Transformed cells have, characteristically, a reduced requirement for these growth factors in order to sustain growth.

Our observations have shown murine sarcoma virus (MSV) transformed cells produce and release growth factors that combine with and interact through the EGF receptor system (DeLarco, J.E. and Todaro, G.J. Natl. Acad. Sci. U.S.A. 75: 4001, 1978, DeLarco, J.E. and Todaro, G.J., J. Cell. Phys. 102: 267, 1980).

There appears to be a family of these peptides, called sarcoma growth factors (SGFs), that are produced and released by the MSV-transformed cells. They are able to compete with ^{125}I-EGF for the EGF receptors and act as potent mitogens for cells that are arrested because of confluence or serum depletion. These SGF peptides are, unlike EGF, able to induce morphological changes in untransformed cells that are phenotypically indistinguishable from the transformed genotype. In the presence of these peptides the cells grow over one another in a criss-cross fashion, form multiple layers and form large colonies in soft agar. If the phenotypically "transformed cells" are reseeded in the absence of SGF they lose the transformed morphology. They now grow as regular monolayer and are no longer able to grow in soft agar. If, however, the SGF is added back to the cultures they again phenotypically express the transformed morphology.

The rationale of the studies reported here is to determine: if the SGFs produced by the MSV-transformed cells are of viral or cellular origin; if their properties are capable of explaining, on a biochemical basis, the morphological phenotype of transformed cells; if these peptides are of cellular origin, are they expressed during development and/or by tissues in the adult organism; and if these peptides are related to the growth factors released by human tumor lines.

Methods Employed:

The source of the sarcoma growth factors is serum-free media that has been conditioned by transformed cells. The cells are seeded and grown to confluency in media containing 10% calf serum. They are then washed twice in serum-free media, which is discarded, and additional 48 hour harvests of conditioned serum free media are collected. This conditioned media is clarified by a low speed and then a high speed centrifugation. The supernate from the high speed centrifugation is concentrated 20-fold using a hollow fiber concentrator (Amicon DC-2). The concentrate is then dialyzed extensively against 1% acetic acid (five changes of 10 vol.) in Spectrapor 3 dialysis tubing (molecular weight cutoff, approximately 3500). The retentate is further concentrated by lyophilization and the acid soluble peptides extracted with one molar acetic acid. The initial step in purification is done using Bio-Gel P-60 gel filtration chromatography. Aliquots of the individual column fractions are assayed for: protein content; EGF competing activity, and soft agar growth stimulating activity. The main peaks of activity are pooled, lyophilized and further purified. The peptides present in these pools are further separated using carboxymethyl cellulose ion exchange column chromatography. Aliquots from these fractions were assayed as above and those fractions containing the activities of interest are pooled and lyophilized. Following ion exchange chromatography the peptides are further separated on reverse phase high performance liquid chromatography (HPLC). The initial HPLC is run on a C column. The bound peptides are eluted from the column using a linear propanol gradient. The active fraction(s) from this column is rechromatographed on a similar column using a linear acetonitrile gradient rather than a propanol gradient to elute the peptides.

Major Findings:

1. Purification of SGF via the EGF receptor. The 10,000 molecular weight protein previously defined sarcoma growth factor (SGF) (DeLarco and Todaro, Poc. Natl Acad. Sci. (USA) 75:4001, 1978) has been radiolabeled using ^{125}I. This radiolabeled peptide was purified using the EGF receptors on formaldehyde-fixed A431 cells. This ^{125}I-labeled peptide was demonstrated to form a sharp peak upon isoelectric focusing with an isoelectric point of 6.8, distinctly different from EGF's 4.4. This radiolabled peptide bound to the cell's EGF receptors and could be completely blocked by an excess of unlabeled EGF. A cellular response to SGF required that the cells express functional EGF receptors. This peptide is similar to EGF in that it is a small, heat and acid stable peptide which requires disulfide bonds for activity and it interacts with the EGF receptor to cause its biological response. It differs from EGF in that it has a much more profound morphological effect on cells, it does not interact with antisera directed against mouse salivary gland EGF, and it has a distinctly different pI.

2. Similarity of SGFs from different cells. The characteristics of the SGF-like growth factors released by different sarcoma virus transformed cells appear to be quite similar. The major SGF-like growth factor from a Kirsten sarcoma virus (KiSV) transformed normal rat kidney clone (KNRK) appears quite similar to the SGF isolated from the Moloney murine sarcoma virus (MSV) transformed murine 3T3 clone (3B11-IC). These peptides appear to be similar in: their biological properties (Fig. 1A), they appear to have similar molecular weights (Fig. 1B), and migration properties during carboxymethylcellulose chromatography (Fig. 2). The elution profiles were similar for these peptides even though the origin of the cells and the transforming viruses were different in these two clones. The results indicate the major growth factor from a murine cell transformed by a murine-derived sarcoma virus is similar in both size and charge characteristics to the major growth factor released by a rat cell transformed by a sarcoma sequence of rat origin. This class of peptides may serve an essential role in growth and/or development as it appears to have been well conserved between mouse and rat cells. Questions concerning the conservation and the function of SGFs may be answered when probes to these factors are available. The results indicate the release of SGF-like peptides by sarcoma virus transformed cells in common to two distinct sarcoma viruses and two different species of recipient cells. Untransformed normal rat kidney cells (NRK), however, did not release detectable quantities of SGF-like growth factors into their serum-free media (Fig. 1C), even though the assays used higher protein concentrations and the criteria for anchorage independent growth were much lower than those for the peptides released by the sarcoma virus transformed cells (i.e. a cluster of cells greater than four would have been scored as a colony in the assays on the peptides released by the untransformed cells, whereas, only colonies with greater than 50 cells were scored as positive in assays on the peptide fractions from the sarcoma virus transformed cells).

3. Origin of SGF gene. Clones of NRK cells transformed by either the wild type Kirsten sarcoma virus (KiSV) or a mutant that is temperature sensitive

with respect to transformation (ts-371 clone 5) (Shih et al., J. Virol. 31: 346, 1979) were examined at the permissive temperature (32°C) and the nonpermissive temperature (39°C) for: expression of available EGF receptors; expression of the transformed phenotype, and production and release of SGF-like peptides. Tables 1 and 2 show the results of these studies. For the data seen in Table 1, the cells were plated and grown for 24 hours at an intermediate temperature (36°C) and then shifted for 24 hours to 32°C, 36°C, or 39°C before assaying for EGF binding. The cells transformed by wild type virus show essentially no receptors at any of the temperatures, the cells transformed by the mutant viruses show a greatly increased number of receptors at 39°C, comparable, in fact, to those found on the untransformed parental cells. At 32°C, however, they show only 10 to 20% the amount of binding seen at 39°C. The cells maintained at the intermediate temperature have repeatedly expressed an intermediate number of available EGF receptors.

Table 2 shows that the ts-371 transformed cells are able to respond to SGF at the nonpermissive temperature. In soft agar assays they are comparable to their untransformed parental cells in their ability to respond to SGF. Serum-free conditioned media from the ts-371 transformed cells were collected at 32°C and 39°C. The EGF competing activity and the growth factor activity present in the conditioned media collected from the cells maintained at 32°C was at least 10-fold greater in the conditioned media collected at 39°C.

To determine if SGF produced by a sarcoma virus transformed cell is a direct product of the sarcoma genome or a secondary product whose expression is controlled by the sarcoma gene product, the SGF-like peptides were isolated from serum-free media conditioned at 32°C by a clone of NRK cells transformed by the ts-KiSV (ts-371 clone 5) and by a clone of NRK cells transformed by the wild type KiSV (KNRK). If the activity released from the cells transformed by the ts-sarcoma virus is itself much more temperature sensitive than that isolated from the media conditioned by wild type virus, this would support a model in which the SGF is a direct product of the ts-sarcoma gene; whereas, if the SGF isolated from the ts-371 conditioned media was not temperature sensitive, it would support a model in which the sarcoma gene product controls the ex-pression of the growth factors rather than acts directly as a growth factor.
The results of the experiments are found in Table 3. In comparing heated and unheated aliquots from ts-371 clone 5 for their ability to stimulate thymidine incorporation, there were little if any differences at either dilution. The soft agar growth stimulating activity was also insensitive to this heat treatment step as performed (samples were heated in a 65°C water bath for 120 min. at pH 7.0). The results from the heat treatment of the equivalent factor obtained from KNRK cells were quite similar indicating the "SGF" obtained from the cell transformed by the ts-sarcoma virus is not more "temperature sensitive", under the conditions tested, than that obtained from the cells transformed by the wild type sarcoma virus.

4. Purification of a larger molecular weight SGF. An earlier eluting peak of EGF competing activity from the serum-free conditioned media of the MSV transformed cell line 3B11-IC has been chosen for further characterization and purification. This is a peptide with an apparent molecular weight of approximately 20,000. It has good EGF-competing activity and is a potent stimulator of ^{3}H-thymidine incorporation. Its ability to stimulate soft agar growth is

less than that of the 10,000 molecular weight peptide that has been called SGF. It, however, is a very potent stimulator of soft agar colony formation. Using four chromatographic columns, we have what appears to be a homogeneous peptide that competes on a mole for mole basis with mouse submaxillar gland EGF. This peptide has been shown to interact with the EGF receptor system on the A431 cell line and in so doing can stimulate the phosphorylation of this receptor in the presence of ^{32}P-ATP. When enough of this peptide has been purified to homogeneity, its amino acid sequence will be determined.

5. Production of EGF-like peptides in a nonvirally transformed murine cell. Embryoid bodies (A6050) from Dr. Leroy C. Stevens were allowed to attach and grow in tissue culture dishes. These cells were cloned and one of these clones, which apparently lacks available EGF receptors was examined for the production of an EGF-like peptide. Chromatography of the serum-free conditioned media over a column of Bio-Gel P-60 indicated this clone produced one predominant growth factor. It has an apparent molecular weight of approximately 25,000. This peptide blocks the binding of ^{125}I-EGF, it stimulates the incorporation of ^{3}H-thymidine into the DNA of quiescent serum-depleted fibroblastic cells, and it stimulates NRK cells to form colonies in soft agar. The elution profile from a Bio-Gel P-60 column is similar to that seen for the largest growth factor released by the MSV transformed murine cells (3B11-IC). Unlike the factor produced from 3B11-IC cells, however, the production of this factor can be modulated by nontoxic doses of retinoic acid and EGF binding to these cells can be stimulated by treating them with retinoic acid. The quantity of EGF these cells will bind depends upon the length of treatment and the dose of retinoic acid used during this treatment. Retinoic acid prevents these cells from growing in soft agar and also decreases the amount of growth factors these cells release into their media.

Significance to Biomedical Research and the Program of the Institute:

This work is significant and of high priority because at a basic research level it seeks to define the mechanisms and factors which are responsible for the uncontrolled proliferation and the phenotypic transformation of malignant cells. At the applied level, it aims to detect and characterize growth or differentiation factors that are responsible for the control of cellular proliferation and differentiation in normal and cancerous tissue. These goals are consistent with the main goals and mission of the NCI and should produce results that will be applicable to patient care in both early detection and treatment.

Proposed Course:

The project will continue in two phases. The first of these will be the purification and sequencing of the major classes of growth factors released by transformed cells, which are able to induce anchorage independent growth in untransformed fibroblastic cells. The sequences of these factors will be compared with those of characterized peptide hormones and growth factors to determine if any homologies exist. This, hopefully, will shed light as to the origin, function and control of these factors that are being ectopically produced and released by the tumor cells. The second phase will use the amino acid sequences to construct nucleotide probes to purify the mRNAs for these factors. Reverse transcribed complementary DNAs from the mRNAs will allow the cloning of these genes. With clones available the transcription and expression of these genes can be monitored during development as well as in neoplasia. Along parallel lines the purified growth factors or synthetic peptides corresponding to known sequences found in the sequenced factors will be used to develop antibodies for radioimmune assays. These assays will be used to determine if these factors or peptides immunologically related to them are expressed either during development or by certain tissues under normal physiological conditions. The level of expression in tumors will be examined and compared with those of homologous normal tissues.

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TABLE 1

Effect of Temperature on the Expression of EGF Receptors on ^{125}I-EGF Binding

Cell clone	Previous day at	EGF Bound
TS371 c15	32°	1,470
	36°	3,940
	39°	10,970
Untransformed NRK	32°	8,700
	36°	9,500
	39°	8,300
KiSV Transformed NRK	32°	350
	36°	200
	39°	260

TABLE 2

Effect of Temperature on the Soft Agar Growth Response

Cell Clone	Growth Temp.	Agar Colony Formation
TS371 c15	32°	>500
	36°	>500
	39°	5
+ SGF (20 μg/μl)	39°	150
NRK	32°	0
	36°	0
	39°	0
+ SGF (20 μg/ml)	39°	70
KiSV Transformed NRK	32°	>500
	36°	>500
	39°	>500

TABLE 3

Effects of Heating on the Biological Activity of Growth Factors Released by Cells Transformed by either a Wild type KiSV or a Temperature Sensitive (ts-371) KiSV.

Source of growth factor	Stimulation of ^{3}H-thymidine incorporation cpm above control		Percent of 49F cells that formed soft agar colonies[1]	
ts-371	dilution		dilution	
	1:4	30,651	1:1	75
	1:16	4,758	1:4	62
ts-371 heated[2]	1:4	28,588	1:1	76
	1:16	6,119	1:4	61
KNRK	1:3	135,721	1:3	70
	1:15	28,892	1:15	52
			1:75	4
KNRK heated[2]	1:3	129,766	1:3	66
	1:15	26,552	1:15	40
			1:75	8

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[1] Colonies were scored 10 days after seeding. Those colonies larger than approximately 20 cells were scored as positive.

[2] Heat treatment was carried out for 120 min. in a water bath maintained at 65°C. The samples were adjusted to pH 7.0 before heating.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05124-04 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Modulation of Gene Expression in Primate Placenta

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kurt J. Stromberg	Senior Surgeon	LVC	NCI
OTHER:	Daniel R. Twardzik	Research Chemist	LVC	NCI

COOPERATING UNITS (if any) R.I. Huot, Dept. of Biology, Catholic Univ., Washington, DC; D.T. Krieger, Div. of Endocrinology, Mt. Sinai Med. Ctr., NY; R.W. Ruddon, Litton Bionetics, Inc. (FCRC), Frederick, MD; J.C. Azizkhan, Univ. of VA School of Med., Charlottesville, VA; K.V. Speeg, Dept. of Med., Vanderbilt Univ., Nashville, TN

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The gene expression for primate retrovirus, human chorionic gonadotropin, and placental growth factors was studied. A radioimmunoassay for the major internal structural protein (p26) from Macaca arctoides type C virus (MAC-1) was used to score antigen expression in rhesus placenta and other fetal organs obtained at various times of gestation. Antigen was detected in 16 of 16 placental specimens but not in 10 other different fetal organs from each of 8 selected animals. The levels of antigen in placenta ranged between 2 and 218 ng/mg protein with a correlation between lower antigen expression occurring with term gestation or parity greater than 10. Cocultivation of isolated trophoblasts from 10 rhesus placentas with 3 indicator cell lines (A549, FEC and CE2Th) led to rapid isolation in FEC of type C rhesus retrovirus in 4 of 10 cases. With all 4 isolates, p26 expression was detected in cell monolayers between 2-5 weeks, and MN^{++}-dependent DNA polymerase activity was evident between 5-9 weeks. Previous isolates from Macaca (MAC-1 and MMC-1) were obtained in single long term experiments (over 7 months). Consequently, primary trophoblasts represent a useful differentiated cell source for isolation of infectious retrovirus from a "low producer" primate species.

Project Description

Objectives:

First, to study type C viral expression in rhesus placental organ and cell culture and to establish conditions whereby endogenous retroviruses might be more readily isolated from primate species. The aim has been to establish that there is a tissue-specific preference for expression of endogenous type C virus in primate placenta, and to apply principles established in the rhesus system to primate species from which retroviruses have not been isolated, including red and African green monkeys and chimpanzees. Second, to identify and characterize the mechanism and modulation of expression of human placental hormones in normal and malignant trophoblasts in vitro. These include the alpha- and beta-subunit of human chorinoic gonadotropin (HCG),ticotropic hormone (ACTH) and B-endorphin, as well as various growth factors.

Methods Employed:

Rhesus retroviral expression is scored by radioimmunoassay (RIA) of MAC-1 p26. Clarified pellets (100,000xg) of cell and organ culture supernatants are screened for DNA polymerase activity, using rAdT synthetic templates. An RIA for alpha and beta subunit secretion of HCG, ACTH, and beta endorphin as biochemical markers of trophoblast function are additional assay procedures. Growth factor secretion from placenta is assessed by stimulation of growth of normal rat kidney cells (NRK) in soft agar.

Major Findings:

1. In vitro growth of human placenta. Optimal methods have been developed for growth of human placenta in organ culture and for isolation of trophoblast cells for growth in monolayer culture. The amount of HCG expression with this improved organ culture system was approximately ten thousand times higher on a per gram basis than that obtained with term placenta in conventional explant culture.

2. Modulation of HGC in normal and malignant trophoblast. Four compounds previously shown to elevate the levels of HCG secretion by JAr cells and other choriocarcinoma cells were employed in modulation experiments: epidermal growth factor (EGF), methotrexate (MTX), 5'-fluoro 2'-deoxyuridine (FUdR), and actinomycin D (AMD). Of these four modulation agents, normal placental cultures responded only to FUdR and AMD, suggesting that the regulation of HCG synthesis differed in the JAr line and in the normal placental cultures. In both the JAr and BeWo lines of choriocarcinoma, inhibition of DNA synthesis by MTX had markedly increased secretion of HCG. Receptors for EGF were present on the JAr cells and were localized to the trophoblast cells of normal placental organ cultures as detected by immunofluorescence. Despite the presence of EGF receptors, the normal placenta did not respond to EGF by significantly increasing its levels of HCG production. The JAr line of choriocarcinoma exhibited a twofold increase in HCG secretion after the addition of EGF. EGF stimulated growth in the JAr cells as measured by protein content of the cultures but did not elevate the incorporation of [methyl- H] thymidine in either the JAr cells or placental organ cultures.

3. Biochemistry of HCG secretion. Initial experiments show a close similarity in the synthesis and secretion of HCG subunits between choriocarcinoma cell lines and normal first trimester placenta in organ culture using pulse-chase labeling experiments and immunoprecipitation and SDS-PAGE of cell lysates. Both contain 15,000 M.W. and 18,000 M.W. precursors of alpha-subunits and secrete a mature alpha-subunit of 22,000 M.W., as well as 18,000 M.W. and 24,000 M.W. precursors of beta-subunits and secrete a mature beta-subunit of 34,000 M.W. Interestingly, however, the kinetics of beta-subunit secretion of HCG in normal placenta are in the manner of a bell shaped curve with peak levels at day 6 in organ culture. In contrast, alpha-subunit secretion is nearly level throughout the 12 day course of culture. Preliminary experiments in collaboration with Dr. Dorothy Krieger indicate the kinetics of secretion of ACTH and beta-endorphin are identical to the kinetics of secretion of the beta-subunit of HCG.

4. Growth factors in primate placenta and amniotic fluid. An acid soluble (pH 2) polypeptide has been identified in conditioned medium from human placental organ cultures, with an estimated M.W. of 30,000, which supports growth of normal rat kidney cells (NRK) in soft agar. Its distinction from a glycoprotein stimulating granulopoiesis (HPCM factor) is under evaluation by several techniques including con A sepharose chromatography. Amniotic fluid from human and subhuman primates in addition to epidermal growth factor also contains an 8,000 M.W. and approximately 17,000 and 30,000 M.W. polypeptides with EGF-competing activity whose capacities to stimulate NRK soft agar growth is presently being tested.

5. Retroviral isolation and antigen expression in primate trophoblast. A radioimmunoassay for the major internal structural protein (p26) from *Macaca arctoides* type C virus (MAC-1) was used to score antigen expression in rhesus placenta and other fetal organs obtained at various times of gestation. Antigen expression was detected in 16 out of 16 placental specimens but not in 10 other different fetal organs from each of 8 selected animals. The levels of antigen detected in placenta ranged between 2 and 218 ng/mg protein with a correlation between lower antigen expression with term gestation or parity greater than 10. A tenfold higher level of antigen expression was detected at the external surface of the placenta near the decidua than in the remainder of the placenta towards the amniotic surface. Thus, even within the placenta, there was a preferred site for endogenous retroviral antigen expression. Lastly, substantial inter-individual variation exists in regard to the amount of placental p26 expression in preterm animals of low (1) or intermediate (2-6) parity. Separate cocultivations of isolated trophoblasts from ten rhesus placentas using three indicator cell lines (A549, FEC and CF2Th) led to rapid isolation in feline embryo cells (FEC) of type C Rhesus retrovirus in four of ten cases. With all four retroviral isolates, p26 expression was detected in the cell monolayers between two and five weeks, and MN^{++}-dependent DNA polymerase activity was evident in the culture supernatants between five and nine weeks after initiation of cocultivation. Five of the remaining six sets of cocultivations grew Simian foamy virus and were discontinued. The two previous isolates from *Macaca* (MAC-1 and MMC-1) were both obtained in single long term experiments (over seven months) after multiple attempts had failed to yield virus. Consequently, primary trophoblast cells represent a useful

differentiated cell source for isolation of infectious retrovirus from a "low producer" primate species.

Significance to Biomedical Research and the Program of the Institute:

The relevance of the retroviral animal model to human cancer can be tested when a bona fide retrovirus of human origin is growing in cell culture in adequate yield. Only then can appropriate detection techniques, such as radioimmune precipitation assays for certain viral polypeptides, be developed to correlate retroviral expression with neoplastic growth in man. This project is in part devoted to this aim by using the rhesus placenta as a model system. As factors which influence retroviral expression, in what may be a physiological manner in rhesus placenta become evident, they will be adapted toward retroviral induction in higher primate species, including man, from which retroviruses have not yet been isolated.

Knowledge of mechanism of modulation of expression of biochemical markers of malignancy, such as HCG and growth factors is essentiial for the markers to be useful clinically. These markers can be easily studied in normal and malignant human placental tissue in cell and organ culture.

Proposed Course:

As an extension of the ease of retroviral isolation in the rhesus system, cocultivation of various indicator lines is underway with isolated trophoblast target cells from *Erythrocebus patas* (red) and *Cercopithecus aethiops* (African green) monkeys, as well as *Pan troglodytes* (chimpanzee). In addition, by using various cloned primate proviral probes, preferential transcription in trophoblast RNA will be monitored in these primates by means of Northern blot hybridization procedures.

With respect to differentiated trophoblast gene products from normal, principally human, placenta in vitro 1) a biochemical characterization of the kinetics and processing of HCG subunits will be carried out, including use of digestion with endoglycosidase H; 2) the equivalence of placental and pituitary ACTH and beta-endorphin will be examined; and 3) characterization will be made of placental and amniotic growth factors following bulk collection of conditioned medium and fluid.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05141-02 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

A Cell Culture Model System for Studying Late-Stage Promotion of Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Nancy H. Colburn	Expert	LVC	NCI
OTHERS:	Louis D. Dion	Staff Fellow	LVC	NCI

COOPERATING UNITS (if any)
W. Laug and W. Benedict, University of Southern California, Los Angeles, CA

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.06	0.30	0.76

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this work has been to further develop and characterize a mouse epidermal cell culture model system for studying late-stage promoter-dependent preneoplastic progression and its prevention. Recently published or in press results have shown that JB6 mouse epidermal cells respond to second stage but not first stage (described by Slaga et al.) tumor promoters to undergo irreversible promotion of anchorage independence and tumorigenicity. Promotion of tumor cell phenotype by phorbol esters is blocked by inhibitors of second stage promotion such as retinoids but not by inhibitors of first stage promotion such as antiproteases. The mechanism of promotion of tumor cell phenotype in JB6 cells appears to involve induction of new phenotype(s) rather than selection of preexisting variants. Clonal derivatives of JB6 which are promotable by phorbol esters are also promotable by other classes of tumor promoters such as ingenols, growth factors, detergents and cigarette smoke. The nonpromotable counterparts have to date shown consistent cross resistance to several classes of promoters suggesting that promotion sensitivity in JB6 cell lines is determined by a pathway common to a variety of promoters.

Project Description

Objectives:

To determine the rate limiting steps which occur during the long latent period of premalignant progression following exposure to carcinogens and or tumor promoters. Use, characterization and modification of the JB6 mouse epidermal cell model system which has been previously described for studying promotion of transformation as measured by anchorage independence and tumorigenecity. Specifically, (1) to obtain from promotable cell lines, clonal cell lines which are resistant to promotion of transformation by the phorbol diester TPA. To ascertain the degree of cross resistance (and cross sensitivity in the TPA-sensitive lines) to other classes of promoters. (2) To determine in this system the activity of various known or postulated antipromoters. (3) To continually characterize the clonal cell lines and monitor them for stability.

Methods Employed:

Characterization and cloning of mouse epidermal cell lines including the determination of anchorage independent growth (colony formation in 0.33% agar) in response to tumor promoters and antipromoters. Establishment of promoter-induced transformant lines cloned from agar.

Major Findings:

1. Promotion resistance (or sensitivity) in JB6 cell lines extends to phorbol esters and several other classes of promoters. Clonal heterogeneity has been found for promotion of anchorage independence in clonal derivatives of the promotable JB6 mouse epidermal line. The clonal lines which are most responsive to phorbol ester promoters are also most responsive to other classes of promoters including ingenols, detergents, polycyclic hydrocarbon derivatives in cigarette smoke, and epidermal growth factor.

2. Retinoids inhibit promotion of anchorage independence in JB6 cells by TPA. Promotion of tumor cell phenotype by the phorbol diester TPA is inhibited by retinoids which also act as antipromoters in mouse skin tumor promotion. The sturcture activity relationship for antipromotion by retinoids in vitro paralled that found in vivo.

3. Promotion of transformation in JB6 cells occurs by induction of a new phenotype. The mechanism of promotion of tumor cell phenotype in JB6 cells involves induction of a new phenotype rather than selection of preexisting anchorage independent variants. This means that the mechanism of late stage promotion of transformation is expected to involve induced (or repressed) synthesis of one or more significant macromolecules.

4. Analogy of JB6 model to second stage promotion (Slaga et al.) in vivo. Promotion of transformation in JB6 cells occurs in response to second stage promoters such as mezerein but not to first stage promoters such as the calcium inophore A23187. Inhibition of the promotion response occurs with

second stage inhibitors such as retinoids but not with first stage inhibitors such as antiproteases.

Significance to Biomedical Research and the Program of the Institute:

The availability of an in vitro model system for studying promotion of transformation by a variety of classes of tumor promoters and its inhibition by various antipromoters is useful both for promoter or antipromoter detection and for mechanism studies. The availability of paired promoter sensitive and resistant cell lines is useful for ascertaining the changes which determine promotion vs. the correlative ones.

Proposed Course:

To extend this work to the study of new promoters and antipromoters. To develop new JB6 cell variants each resistant to one TPA response, which will be useful for ascertaining the role of the response in the mechanism of promotion of tumor cell phenotype. To develop a human cell model system to study promotion of transformation.

Publications:

Colburn, N.H., Koehler, B., and Nelson, K.A.: A cell culture assay for tumor promoter dependent progression toward neoplastic phenotype: Detection of tumor promoters and promotion inhibitors. Teratogenesis, Carcinogenesis, Mutagenesis 1: 87-96, 1980.

Colburn, N.H.: Tumor promoter produces anchorage independence in mouse epidermal cells by an induction mechanism. Carcinogenesis 1: 951-954, 1980.

Colburn, N.H., Ozanne, S., Lichti, U., Ben, T., Yuspa, S., Wendel, E., Jardini, E., and Abruzzo, G.: Inhibition of promoter dependent preneoplastic progression by retinoids. Annals NY Acad. Sci. 359: 251-259, 1981.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05143-02 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Pathogenesis of Virus-Associated Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kamaraju Sreemahalakshmi	Visiting Fellow	LVC	NCI
Other:	Paul Levine	Medical Director	LVC	NCI
	Roger Connelly	Research Statistician	BB	NCI
	Costan Berard	Pathologist	LP	NCI
	Larry Muenz	Mathematical Statistician	BB	NCI
	Jose Costa	Pathologist	LP	NCI

COOPERATING UNITS (if any)

N. Mourali, Institute Salah Azaiz, Tunis, Tunisia, and R. Dorfman, Stanford Univ. Med. Center, Stanford, CA.

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Clinical Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.95	0.70	0.25

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Information was collected relating to the clinical features, pathologic features and hormone receptor status of breast cancer patients in Tunisia. Evidence for the unusual progression of disease in Tunisian breast cancer patients was documented with the finding of a poorer nuclear grade in RPBC patients than non-RPBC patients. In contrast to American patients with inflammatory breast cancer, who are reported to have low levels of hormone receptors, patients with RPBC had levels comparable to non-RPBC breast cancer patients. Information collected on 256 pathologically confirmed cases of Burkitt's lymphoma (BL) diagnosed in the Western hemisphere demonstrated the curability of BL, particularly in patients with limited disease. Target organs appeared to be related to age, suggesting a predilection of the disease for rapidly dividing cells. Laboratory and epidemiologic studies provided evidence for environmental as well as genetically determined predisposing factors. Patients with Epstein-Barr virus (EBV)-associated tumors appeared to have a longer survival rate than those with non-EBV-associated tumors.

Project Description

Objectives:

The purpose of this project is to correlate clinical, epidemiologic and pathologic findings in patients with virus-associated tumors with the subsequent attempt to relate these parameters to virologic assays.

Methods Employed:

Information was collected from more than 100 patients with breast cancer followed at the Institute Salah Azaiz in Tunis, Tunisia. Information from three separate studies, a chemotherapy study involving 112 patients, a pathology study involving 102 patients, and a hormone receptor study involving 94 patients, were integrated with an attempt to interrelate all of the clinical, pathologic and laboratory values. The American Burkitt Lymphoma Registry was maintained and cases continued to be collected from the Clinical Center at NIH, from the literature, and from personal contact with various physicians from different parts of the country. The patients' physicians were contacted for clinical information, diagnostic blocks, and sera. The diagnostic blocks were sent to Dr. Berard (NIH) for review and confirmation of the diagnosis. The sera obtained from these cases were tested for antibody titers to the Epstein-Barr virus (EBV) viral capsid antigen (VCA) and early antigen (EA). The data from 256 Burkitt's lymphoma (BL) patients were then analyzed for various racial, geographical and epidemiological patterns and compared to the African patterns.

Major Findings:

1. Characterization, pathogenesis and control of rapidly progressing breast cancer in Tunisia. Rapidly progressing breast cancer (RPBC) or pousee evolutive (PEV) proved to be an entity with certain pathologic features, such as poor nuclear grade and involvement of the dermal lymphatics with tumor microemboli. The evaluation of hormonal levels in Tunisian breast cancer cases showed increased estrogen receptor (ER) and progesterone receptor (PR) levels in comparison to American patients with inflammatory breast cancer. No difference was seen in the hormone receptor patterns between RPBC and non-RPBC patients. ER and PR levels were lower in pre- than post-menopausal patients. CMF proved to be effective in controlling RPBC. Forty-two percent of patients showed a complete response to CMF, 54.7% had a partial response (more than 50% decrease), and an additional 17% showed objective improvement. Of all the factors relating to response to CMF, stage of disease proved to be most important. No difference was seen between surgery and radiotherapy as an adjunct to chemotherapy except in patients with advanced disease (PEV-3) who responded better to radiation therapy.

2. Burkitt's lymphoma in the United States. On evalution of data from the American BL Registry, it was noted that the major characteristics of American BL patients tended to be male predominance, a young age group involvement (4-12 yrs.), and a higher incidence in Caucasians. More recently older patients have been frequently reported, perhaps due to better

awareness of the disease on the part of non-pediatricians. In contrast to the earlier report showing a paucity of patients in the high altitudes, cases were obtained from all parts of the country. A positive cancer history was obtained in first degree relatives of 18 of the 152 cases on whom detailed pedigrees could be obtained. Abdominal viscera were affected most often, the most outstanding being the ileum. Unlike African cases, cervical lymph nodes were also among the most common organs to be involved. Better median survival rates for Stage I and II cases were observed than for patients with Stage IV disease. No relapse was observed in American cases beyond 2 years, whereas 14 late relapses were observed in a series of 117 African patients. The survival seemed to be prolonged in patients with elevated VCA antibody titers. In contrast to African BL patients, Americans had more older patients, less EBV-positive patients, and fewer patients with jaw tumors.

Significance to Biomedical Research and the Program of the Institute:

In order to determine the significance of certain viral markers in human cancer, it is important to have well-characterized specimens from patients who have been appropriately studied. This project provides the characterization of patients with two diseases under close study by the National Cancer Institute. The Tunisian breast cancer patients are of importance because they have an unusually aggressive disease and provide an opportunity to study factors which promote tumor and which limit their growth, the information also being applicable to breast cancer in the United States. The studies on Burkitt's lymphoma are of importance because BL is one of the tumors with the most prominent epidemiologic and laboratory evidence indicating a viral etiology. Two major cofactors implicated in the cause of BL are malaria and the Epstein-Barr virus. Studies on BL in an area where malaria is absent help to clarify the role of EBV in the pathogenesis of this disease.

Proposed Course:

Groups of breast cancer patients from different parts of the world will be studied in order to evaluate their clinical, pathologic, epidemiologic, and immunologic features. Particularly, the antigen cross-reacting with gp52 of the mouse mammary tumor virus that has been identified in biopsies from Tunisian breast cancer patients will be evaluated in biopsies from patients coming from Eygpt and other parts of the world. The studies on Burkitt's lymphoma and other EBV-related tumors will be pursued in the laboratory to detect EBV related markers in patient tissues and immune reactivity to EBV in patients with BL and similar lymphomas.

Publications:

Levine, P.H., Kamaraju, L.S., Connelly, R.R., Berard, C.W. Dorfman, R.F., Magrath, I., and Easton, J.M.: The American Burkitt's Lymphoma Registry: eight years' experience. Cancer, in press.

Costa, J., Webber, B.L., Levine, P.H., Muenz, L., O'Conor, G.T., Tabbane, F., Belhassen, S., Kamaraju, L.S., and Mourali, N.: Histopathological features of rapidly progressing breast cancer in Tunisia. Int. J. Cancer, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05144-02 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Polypeptide Growth Factor-Induced Alterations in Proliferating Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ursula Heine	Research Microbiologist	LVC	NCI
Other:	Jorma Keski-Oja	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)
B. Wetzel, Dermatology Branch, NCI, Bethesda, MD.

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.16	0.65	0.51

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to evaluate the mechanisms of action of growth-promoting polypeptide hormones. The effects of epidermal growth factor (EGF) were evaluated by scanning electron microscopy in human and murine epithelial cells and were found to be reversible, dose-dependent, and dependent on the number of surface receptors. Plasma membrane mobility, macropinocytosis, and cell proliferation were enhanced. Comparative studies revealed that epithelial cells of different origin respond differently to EGF, and that membrane ruffling followed by pinocytosis was the prevalent, early specific morphologic response. This response was clearly defined in murine epithelial cells, strain MMC-E, thus making these cells the preferred model for such studies.

Project Description

Objective:

To define the mechanisms of action of epidermal growth factor with respect to growth and morphology of cultured epithelial cells of human and murine origin.

Methods Employed:

Tissue culture techniques, phase and interference contrast light microscopy, scanning electron microscopy, tracer studies.

Major Findings:

Characterization of rapid membrane changes in epithelial cells after exposure to epidermal growth factor. We have characterized, by use of scanning electron and light microscopy, the short term effects of epidermal growth factor (EGF) on nontransformed murine MMC-E cells and compared them with the response of the human epidermoid carcinoma A431 cells. We clearly demonstrated that EGF initiates a well-defined series of events at the surface of MMC-E cells. Within seconds after the addition of medium containing 100 ng/ml EGF, the MMC-E cells uniformly develop marginal surface ruffles in association with macropinocytosis, and these activities subside within one hour. In contrast, A431 cells respond less uniformly, and the addition of fresh, prewarmed medium lacking EGF elicits similar changes. We also characterized, by use of scanning electron microscopy and light microscopy, the short term effects of epidermal growth factor (EGF) (100 ng/ml) on murine MMC-E epithelial cells and compared them with the response of the human epidermoid carcinoma A431 cells. We clearly demonstrated that EGF initiates a well-defined series of events at the surface of MMC-E cells. Ruffles appear peripherally on many of these cells and migrate within minutes towards a more central location at the cell surface where most endocytic vacuoles are formed. This process begins immediately after addition of EGF and subsides after one hour. In contrast, A431 cells respond less uniformly to the same concentration of EGF, and the addition of fresh, prewarmed medium lacking EGF elicits similar changes. The more uniform response of MMC-E cells to EGF, and the relative insensitivity of this cell line to the addition of fresh medium should facilitate future studies on the molecular mechanisms underlying the response of nontransformed epithelial cells to this and other growth factors.

Significance to Biomedical Research and the Program of the Institute:

The majority of malignant tumors are represented by carcinomas; yet, most of the models developed for the study of transformation and malignancy consist of cells derived from the mesoderm. Our studies revealed that epithelial, nontransformed murine cells grown in vitro respond extremely rapidly to EGF and this response has a distinct pattern of events. Thus, this cell system is extremely useful as a model to test a variety of other growth factors, especially those provoking transformation. Moreover, the system is now available to serve as a model for the study of plasma membrane motility, a phenomenon that remains as yet completely obscure.

BEST DOCUMENT AVAILABLE

Proposed Course:

None. This project has been completed.

Publications:

Keski-Oja, J., Heine, U.I., Rapp, U.R., and Wetzel, B.: Epidermal growth factor-induced alterations in proliferating mouse epithelial cells. Exp. Cell Res. 128: 279-290, 1980.

Lauter, C.J., Heine, U., and Trams, E.G.: Ecto-enzymes on vesicles released from cultured CNS cells. Biochem. Biophys. Acta, in press.

Heine, U.I., Keski-Oja, J., and Wetzel, B.: Rapid membrane changes in mouse epithelial cells after exposure to epidermal growth factor. Exp. Cell Res., in press.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05149-02 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Tumor Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mohammed E. Shoyab	Expert	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.20	1.10	1.10

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project aims to isolate and characterize putative endogenous tumor promoters and to develop a rapid and economical assay for identification of exogenous and endogenous tumor promoters. The following activities were specifically pursued: (1) elucidation of the mechanism of 12-O-tetradecanoyl-phorbol-13-acetata (TPA)-induced inhibition of epidermal growth factor (EGF) binding (TIEB) and search for a compound(s) which can reverse and modulate TIEB at nontoxic doses; (2) isolation and characterization of membrane receptors of phorbol esters; (3) search for endogenous ligand(s) for phorbol receptors, and their isolation and characterization; (4) isolation and characterization of growth factor(s) induced by biologically active phobol esters; (5) test whether EGF and other growth factors enhance carcinogenesis in vivo and in vitro; (6) investigate whether TPA induces or enhances the expression of endogenous oncogenic cellular information; (7) study whether retroviruses act as promoters in carcinogenesis initiated by chemical or physical agents; (8) reversal of anchorage independent growth of transformed cells by differentiation inducing agents; (9) isolation and characterization of TPA binding protein; (10) study of the catabolism of phorbol diesters.

PHS-6040
(Rev. 10-76)

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Project Description

Objectives:

The process of tumor induction has been broadly divided into two stages, i.e., initiation and tumor promotion. The initiation process apparently involves irreversible alteration in the genetic material whereas tumor promotion appears to be epigenetic in nature and is reversible at least in the early stages. Hence, the interruption of the carcinogenic process should be feasible at the tumor promotion stage. This project aims to isolate and characterize putative endogenous tumor promoters and to develop a rapid and economical assay for identification of exogenous and endogenous tumor promoters. The following activities were specifically pursued: (1) elucidation of the mechanism of 12-O-tetradecanoyl-phorbol-13-acetata (TPA)-induced inhibition of epidermal growth factor (EGF) binding (TIEB). To search for compound(s) which can reverse and modulate TIEB at nontoxic doses; (2) isolation and characterization of membrane receptors of phorbol esters; (3) search for endogenous ligand(s) for phorbol receptors, and their isolation and characterization; (4) isolation and characterization of growth factor(s) induced by biologically active phorbol esters; (5) test whether EGF and other growth factors enhance carcinogenesis in vivo and in vitro; (6) investigation of whether TPA induces or enhances the expression of endogenous oncogenic cellular information; (7) study whether retroviruses act as promoters in carcinogenesis initiated by chemical or physical agents; (8) reversal of anchorage independent growth of transformed cells by differentiation inducing agents; (9) study of metabolism of phorbol diesters in vitro and in vivo.

Methods Employed:

Tumor promoters are a specific class of co-carcinogens which themselves are noncarcinogenic but which can induce tumors in animals previously treated with a suboptimal dose of certain chemical carcinogens. Among the most potent of these compounds are plant diterpenes including 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA and other biologically active phorbol esters elicit and modulate a variety of biochemical and biological functions in mouse skin including stimulation of macromolecular synthesis, histone phosphorylation, synthesis of phospholipids and modulation of the metabolism of polyamines and cyclic nucleotides. In addition, these compounds induce ultrastructural changes in and affect the differentiation of murine epidermis. Tumor-promoting phorbol esters also evoke pleiotypic responses in cultured cells, including stimulation of macromolecular synthesis and cell proliferation, induction of plasminogen activator and ornithine decarboxylase, loss of surface-associated fibronectin, alterations in the metabolism of cyclic nucleotides and polyamines, stimulation of prostaglandin synthesis, either the inhibition or stimulation of differentiation, alterations in cell morphology and permeability, and elevation in the level of Na -K ATPase activity. Several biochemical and biological studies provide evidence that the initial site of action of tumor-promoting phorbol esters may be the membrane of target cells. The tumor-promoting phorbol esters have been found to modulate the interaction between epidermal growth factor (EGF) and its membrane receptors in a variety of cells in culture. The pleiotypic effects of TPA and the related tumor promoters in vivo as well as in vitro mimic several actions of growth stimulating polypep-

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tide hormones such as EGF and sarcoma growth factor (SGF). We have formulated the working hypothesis that TPA and certain analogues may have some structural resemblance to the endogenous growth-promoting and differentiation modulating substance(s) that have specific membrane receptors. TPA recognizes and interacts with these receptors, thus mimicking the action of putative substance(s). Furthermore, TPA can induce and regulate the production of these endogenous substance(s). TPA can also increase cell susceptibility to a normal regulator of cell growth and modulator of cell differentiation.

Major Findings:

1. Affinity modulation of epidermal growth factor membrane receptors by biologically active phorbol esters. TPA reversibly inhibits binding of I-labeled EGF to treated normal mouse, human, mink, cat, rabbit, hamster, rabbit and chicken cell lines as well as DNA virus transformed or chemically transformed murine cells. TPA does not affect the binding of concanavalin A (Con A), multiplication stimulating activity, (MSA) insulin, murine retrovirus glycoprotein (gp70), nerve growth factor (NGF), alpha-2-macroglobulin (-2-M) and low density lipoprotein (LDL) to their respective membrane receptors. It alters the affinity of the receptors for EGF without changing the total number of EGF receptors per cell. The Scatchard plots of EGF binding gave curvilinear plots in all the cells investigated suggesting either heterogeneity of receptor populations or negative cooperativity. TPA inhibition of EGF binding (TIEB) is a temperature-, dose- and time-dependent phenomenon. TIEB is completely abolished at 4^{0}C. TIEB is also not exhibited with fixed cells or isolated plasma membranes. Thus, it appears that TIEB is related to the membrane fluidity and those treatments which affect the transmembrane mobility of receptors also alter the TIEB. A puzzling feature of TIEB is that if cells are continuously exposed to TPA for a longer time the degree of TIEB starts to gradually decrease after a few hours and cells become almost refractory after a few days. This loss of TIEB is not due to conversion of TPA to an inactive form because if TPA-containing media from these cells is transferred to new cells, an 85-90% inhibition of EGF binding is observed. TIEB is not significantly affected by steroid hormones, nonsteroid anti-inflammatory drugs, mouse interferon, cytoskeleton disrupting agents, modulators of polyamine metabolism, protease inhibitors, inhibitors of DNA, RNA and protein syntheses, various mucopolysaccharides, water soluble vitamins, vitamins D, E and K1. However, it is partially reversed by retinoids and oubain (Na^{+}-K^{+} ATPase inhibitors). Those phorbol esters (Tigliane, Ingenane and Daphnane Types) which stimulate cell growth in culture and have tumor-promoting activity in vivo in the two-stage tumorigenesis model alter the EGF receptor affinity, while the biologically inactive derivatives fail to change the affinity of the EGF receptor interaction. Rapid assays are needed for tumor-promoting agents both of exogenous and endogenous origin. The specificity, sensitivity and rapidity of TIEB might provide a means for the qualitative and quantitative detection of other classes of tumor-promoting agents.

2. Perturbation of membrane phospholipids alters the interaction between epidermal growth factor and its membrane receptors. To determine the chemical environment of EGF-receptor sites in the plasma membrane and the role of cell membrane lipids, we investigated the consequence of exposure of cells in culture to various hydrolytic enzymes and certain lipid disrupting agents to EGF-

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receptor interactions. Phospholipase C, like the tumor-promoting phorbol esters, specifically inhibits the binding of ^{125}I-labeled EGF to treated mink lungs and murine 3T3 cells but does not reduce the binding of various other ligands to their membrane receptors. Phospholipase C digestion decreases the affinity of the receptors per cell. Certain other agents that interact with membrane phospholipids mimic the phospholipase C effect. The results suggest a role for membrane phospholipids in the EGF-receptor interaction and TIEB may involve the perturbation of the organization of membrane phospholipids.

3. Vitamin K3 and related quinones modulate the affinity of epidermal growth factors for its receptors. The effects of vitamin K3, quinones; fat soluble vitamins and various naturally occurring and synthetic compounds on the binding of ^{125}I-EGF to mink lung cells or murine 3T3 cells in culture was studied. Vitamin K3, but not other fat soluble vitamins markedly inhibits the binding of ^{125}I-labeled EGF to treated cells, but does not affect the binding of insulin, Con A, alpha-2-macroglobulin and murine gp70, to their membrane receptors. The binding of MSA to treated cells is also reduced to some extent. Vitamin K3 alters the affinity of the receptors for EGF without changing the total number of available receptors per cell. Vitamin K3 modulation of EGF-receptor interaction is a temperature-, dose-, and -time dependent phenomenon. EGF-receptor interaction is also significantly modulated by 1,4-napthoquinone, 1-4, benzoquinone and phenanthrenequinone but not by other quinones of anthracyclin antibiotics. Experiments are in progress to test whether vitamin K3 and related quinones act as tumor promoters.

4. Rapid release of fibronectin from human lung fibroblasts by biologically active phorbol esters. A sensitive radioimmunoassay technique has been used to study the effects of several phorbol esters on their ability to release fibronectin from cultured human lung fibroblasts into medium. The biologically active phorbol esters studied rapidly released fibronectin from cells in medium, with concomitant changes in cellular morphology within two hours. The quantity of fibronectin released was dose-, time- and promoter-dependent. The earliest release of fibronectin was seen within 30 minutes of onset of the incubation. Alterations in membrane topology elicited by phorbol esters appear to be responsible for the rapid release of fibronectin molecules from cells into the medium.

5. Specific high affinity receptors for biologically active phorbol and ingenol esters. We have used 20-^{3}H-phorbol-12,13-dibutyrate (PDBu) to study the specific binding of phorbol and ingenol esters and mezerein to a variety of cells in culture and to tissues. ^{3}H-PDBu binds to a variety of normal and trans-formed avian and mammalian cells (mouse, rat, cat, mink, hamster, rabbit, bat, dog, monkey, and human) in a specific, saturable and reversible manner. The specific binding sites are also present in murine brain, spleen, thymus, lung, skin, kidney, heart, stomach, thigh muscle, liver and intestine in decreasing order. Neither human nor mouse erythrocytes bind detectable amounts of ^{3}H-PDBu. Brain and spleen from all strains of mice studied bind exceptionally high amounts of PDBu. The binding increases progressively with age up to one month and remains almost the same until old age. The specific binding of labeled PDBu to live or glutaraldehyde-fixed cells is dose-, time- and temperature-dependent. The optimum binding of PDBu is seen at a pH 6.7.

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The binding is not significantly affected by hydroxyurea (10 mM) actinomycin D (10 μg/ml), cycloheximide (10 μg/ml) and sodium fluoride (1 mM). At saturating concentrations of PDBu, approximately 2 x 10^5 molecules bind to mink lung cells and 5 x 10^5 molecules to Balb/3T3 cells. The apparent K_d values have been found to be 1.3 x 10^{-9} M for mink lung cells and 0.9 x 10^{-9} M for Balb/3T3 cells. Those phorbol or ingenol esters which stimulate cell growth in culture and have tumor-promoting activity in vivo inhibit the binding of labeled PDBu, while the biologically inactive derivatives fail to do so. I.D. 50 (dose for 50% inhibition of binding) values for the PDBu binding and for indirect effect on EGF binding for the various diterpenes correlated very well with each other and with their tumor-promoting potentials. Other nonditerpene tumor-promoting agents, such as phenol, iodoacetic acid, iodoacetamide, bile acids, barbituate, oleate, laurate, limonene, canthradin, anthrodin, saccharin or cyclamate do not affect the binding of ^{3}H-PDBu to its receptors up to a concentration of 10 μg/ml. However, anthralin actually enhances the binding of labeled PDBu to mink lung cells. In addition, cholera toxin, diptheria toxin, oxytocin, vasopressin, gramacidin, monesin, melittin, digitonin, fillipin, amphotricin, kanacidin, ganglioside, nystatin, cholesterol and lysophosphocholine, up to a concentration of 10 μg/ml, do not significantly affect ^{3}H-PDBu-membrane receptor interaction. Also, epidermal growth factors, insulin, retinoids, steroid hormones, prostaglandin, cyclic nucleotides and disulfiram do not compete for the binding of labeled PDBu to its receptors. Certain tissue extracts and body fluids contain factor(s) which modulate the binding of PDBu to its receptors. These data suggest that the biological effects of diterpene tumor promoters are probably mediated through these high affinity receptors. We propose that TPA and active analogues have some structural resemblance to the endogenous growth promoting and/or differentiation modulating substance(s) that have specific membrane receptors. These compounds recognize and interact with these receptors, mimicking the action of putative substances.

6. Partial purification and characterization of a binding protein for biologically active phorbol and ingenol esters from murine serum. We have purified a protein (~Mr 71,000) from murine serum 104-fold. It binds directly biologically active phorbol and ingenol esters, and mezerein in a specific, reversible and saturable manner. The binding of labeled phorbol-12,13-dibutyrate to protein is rapid and dose-dependent. Those phorbol and ingenol esters which stimulate cell growth in culture and have tumor-promoting activity in vivo inhibit the binding of labelled PDBu, while the biologically inactive derivatives fail to do so. Other nonditerpene tumor promoters, retinoids, steroids and prostaglandins do not interfere in PDBu-protein interaction. EGF, insulin, bovine serum albumin, hemoglobin, ovalbumin, ferritin, myoglobin, fetuin and lipase do not directly interact with PDBu. Binding protein competitively inhibits the binding of PDBu to specific receptors. It is a nonglycosylated, slightly hydrophobic protein which is heat- and acid-labile. The protein is present in sera of various mammalian species. The concentration of protein in murine serum is age-, sex-, and strain-independent.

7. Isolation and characterization of phorbol-12,13-diester 12-ester hydrolase (PDEH) from murine and human liver. A phorbol-12,13-diester 12-ester hydrolase (PDEH) has been purified to electrophoretic homogeneity from murine liver cytosol using ammonium sulfate fractionation, sephadex G-200 gel filtration,

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con A sepharose chromatography and phenyl sepharose chromatography. The enzyme is a single chain hydrophobic glycoprotein and it has a molecular weight of 60,000. The enzyme exhibits optimum activity at pH 7.5-8.5. PDEH has an isoelectric point (PI) of 5. The enzyme is heat- and acid-labile. Zn^{++}, Co^{++} and Fl$^-$ inhibit the enzyme. Phenylmethyl sulfonyl fluoride (PMFS) is a potent inhibitor of PDEH. Sarkosyl also inhibits the enzyme at mM concentrations. The enzyme inactivates biologically active phorbol 12,13-diesters in a dose-time- and temperature-dependent manner. The inhibition constant has been observed to be 6.6 x 10^{-8} M for the enzyme-elicited inhibition of phorbol,12-13 dibutyrate binding to its receptor. The enzyme exclusively cleaves the 12-ester of phorbol -12,13-diesters.

8. Phorbol-12,13-diester 12-ester hydrolase (PDEH) as the critical factor in the susceptibility of skin to the tumor-promoting action of phorbol diesters. An esterase, phorbol-12,13-diester hydrolase (PDEH), which converts biologically active phorbol-12,13-diesters to the inactive phorbol-13-monoester is absent from mouse skin but is expressed at high levels in hamster, rat, guinea pig, and rabbit skin. The nonresponsiveness to TPA and related compounds of species other than mouse is directly related to the level of this enzyme found in the skin. One would expect that TPA could act as a potent tumor promoter for human skin since, like mouse skin, it lacks this esterase activity.

9. Certain neuroleptic and antipsychotic tricyclic drugs competitively inhibit interaction between tumor promoting phorbol esters and their specific receptors. Certain antipsychotic drugs such as fluphenazine, chlorpromazine, clopenthioxal, 2-chloroimpiramine and impiramine competitively decrease the binding of ^{3}H-PDBu to its specific receptors. We find a good correlation between PDBu binding inhibiting activity of phenothiazines and impiramine and their biological potency. These results suggest that these widely used drugs might be tumor promoters.

Significance to Biomedical Research and the Program of the Institute:

The EGF-competing activity of the phorbol esters parallels tumor-promoting activity in vivo. The phorbol derivatives lacking tumor-promoting activity also lack EGF-competing activity. TPA treatment seems to modulate EGF binding by decreasing the affinity of the receptors on the treated cells for EGF, rather than by decreasing the number of receptors per cell. This affinity modulation is reversible and dependent on time, temperature and TPA concentration. The effect appears to be specific for the EGF receptor system, as four receptor-ligand systems tested in the same TPA-treated cells and three receptor-ligand systems in the other cells did not show any alterations in receptor affinity. TPA modulation of EGF binding is observed with doses of promoter comparable to those required to elicit biological response in vivo as well as in vitro. The above data suggests that TPA-mediated alterations in growth factor(s)-receptor interaction might be related to the underlying mechanism by which tumor-promoting agents initiate a chain of events causing alteration in cellular growth and function. Interestingly, EGF has been reported to enhance tumorigenesis induced by chemical carcinogens. SGF produced by mouse sarcoma virus-transformed cells also interacts with EGF receptors, stimulates cell

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growth and anchorage-independent growth. The putative endogenous growth factor(s) produced in response to the exposure of cells to tumor-promoting agents, may then, activate a program of gene expression in those cells that have already been genetically altered by initiating agents.

Proposed Course:

This project will continue and extend the findings described to better define the mechanisms of tumor promotion. There is need for rapid cell culture assay for tumor-promoting agents both of exogenous and endogenous origin. The data presented here, showing the consequences of promoter treatment on EGF-receptor interactions and the specificity, sensitivity and rapidity of this response, might provide a means for the qualitative and quantitative detection of other classes of tumor-promoting agents. The isolation, characterization and mode of action of putative endogenous growth and differentiation modulating substances (endogenous tumor promoter[s]) should provide useful information in understanding the mechanism of growth, differentiation and carcinogenesis. These studies have potential to provide clues for designing strategies to reverse and suppress the process of neoplastic development.

Publications:

Shoyab, M., and Todaro, G.J.: Vitamin K3 (Menadione) and related quinones, like tumor-promoting phorbol esters, alter the affinity of epidermal growth factor (EGF) for its membrane receptors. J. Biol. Chem. 255: 8735-8739, 1980.

Shoyab, M., and Todaro, G.J.: Specific high affinity cell membrane receptors for biologically active phorbol and ingenol esters. Nature 288: 451-455, 1980.

Shoyab, M., and Todaro, G.J.: Perturbation of membrane phospholipids alters the interaction between EGF and its receptors. Arch. Biochem. Biophys. 206: 222-226, 1981.

Shoyab, M.: Modulation of the binding of DNBA to DNA of murine epidermal cells in culture by some vitamins. Oncology 38: 187-192, 1981.

Todaro, G.J., De Larco, J.E., and Shoyab, M.: Epidermal growth factors (EGF) receptor interact with transforming growth factors (TGFs) produced by certain human tumor cells and are distinct from specific receptors for phorbol and ingenol esters. Hecker, E. (Ed.): Carcinogenesis, A Comprehensive Survey, Raven Press, New York, in press.

Shoyab, M. and Lubiniecki, A.S.: Thymidine uptake and incorporation in defective in cultured fibroblasts from Fanconi anemia patients. Human Genetics, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05150-02 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Chemical Carcinogenesis and Cocarcinogenesis In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ulf R. Rapp	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)
Dr. Jorma Keski-Oja, Department of Pathology, University of Helsinki,
Haartmaninkatu 3, SF-00290 Helsinki 29, Finland

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS: 1.30	PROFESSIONAL: 0.35	OTHER: 0.95

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An in vitro system was established for the quantitative chemical transformation of mouse epithelial cells mus musculus castaneous epithelial (MMCE). These cells can also be transformed by C3H/MuLV in conjunction with 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Transformed cell clones obtained from soft agar generally were virus nonproducers. The purpose of these experiments is to sequence-label TPA-promotable cellular tumor genes and thus make possible their isolation by molecular cloning.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

Development of improved in vitro assays for chemical transformation of fibroblastic and epithelial cells. Use of co-carcinogenesis for the isolation of new cell-derived tumor genes after linkage with type C viral sequences. Specifically: (1) Develop cell substrates for chemical transformation of a variety of differentiated cells in vitro. (2) Improvement of assay systems to allow processing large numbers of carcinogen-treated cells and development of transformation experiments in a shorter period of time. (3) Use of co-carcinogenesis for the isolation of efficiently transforming type-C viruses or type-C viruses linked cellular tumor genes in culture. (4) Isolation of growth-promoting and transforming factors from chemically transformed epithelial cells using non-transformed epithelial cells as test cells.

Methods Employed:

During the past ten years several lines of evidence have developed to indicate a possible interaction between chemical carcinogens and endogenous RNA viruses. Most of this work has been done in vivo where it is difficult to sort out the relative contributions of transformation frequency versus the host immune response to the overall tumor incidence. We have therefore concentrated on developing quantitative in vitro systems for chemical carcinogenesis. Since most human neoplasia arise in epithelial tissues, emphasis was placed on establishing epithelial cells. Drawbacks of chemical transformation assays in vitro are the required large size of the experiments (low transformation frequencies) and the long time (4-6 weeks) for completion. We developed conditions which allowed assay of large numbers of treated cells within 1 to 2 weeks. To facilitate the generation of new transforming type-C viruses both in vivo and in vitro we examined the effect of carcinogen treatment. Based on our previous observations that carcinogen/mutagen treatment in vivo occasionally shortened the latency period with which C3H MuLV induced tumors, such tumors were established in vitro and type-C virus isolations techniques were applied with good results.

Major Findings:

1. Use of the epithelial and fibroblastic mouse cell lines MMC-E/MMC-F for the determination of the target cell specificity of sarcoma growth factor (SGF). The effects of epidermal growth factor (EGF) on the growth and morphology of mouse embryo epithelial cells (MMC-E) were studied in culture. Growing cultures of epithelial cells were incubated in media containing EGF or certain other mitogenic peptides. It was found that nanogram quantities of EGF stimulated growth in these cells and caused reversible phenotypic changes. These changes were not observed in cultures treated with the other mitogens. The compact growing islands of MMC-E cells were surrounded by elongated border cells (Rapp et al., Cancer Res. 39: 4111, 1979). EGF induced the elongated border cells to flatten and spread. The change of the elongated border cells into polygonal, flattened cells was dependent on the dose of EGF. After treatment with higher concentrations of EGF, all cells appeared more flattened and their cytoplasm was more granular than that of the controls. Scanning electron microscopic studies showed that the elongated border cells in the control cultures were

distinctly higher than the cells inside the islands, while after exposure to EGF they flattened and had fewer surface microvilli than control cells. When EGF was removed and the cells were further cultivated in media without EGF, the border cells became smaller and elongated, eventually resembling those in the control cultures. These results show that EGF may act as a regulatory factor in the control of the proliferation and differentiation of mouse epithelial cells.

2. Effect of murine sarcoma growth factor (SGF) on the growth and morphology of cultured mouse epithelial cells. The MMC-E cell line was also used to study the effects of purified murine sarcoma growth factors (SGFs) on the growth of epithelial cells. Murine SGF was a partially purified preparation from the serum-free culture media of mouse fibroblasts transformed by Moloney murine sarcoma virus. SGF stimulated DNA-synthesis in resting cells and induced them to grow to higher densities than control cells. With continued exposure to SGF, MMC-E cells lost the postconfluency inhibition of division and formed expanding foci. When the SGF was removed and the cells were subcultured, they regained their normal phenotype showing that the effects of SGF are reversible for these cells. SGF could also stimulate these cells to grow in soft agar, like the syngeneic fibroblasts (MMC-F) from the same mouse embryo, but slower than the control fibroblastic clone. Microgram quantities of SGF were needed to stimulate soft agar growth of MMC-E cells. These results indicate that SGF can bring about a phenotypic change in the growth pattern of epithelial cells as well as fibroblastic cells.

3. Transformation of cells in culture by MuLV and TPA. MuLV does not carry a tumor gene but is capable of indirectly transforming cells in vivo. The mechanism by which this occurs occasionally leads to the formation of transmissible new transforming virus and more commonly to linkage of a potential cellular tumor gene with part of the MuLV genome (Payne, et al., 1981; Neel, et al., 1981; Hayward, W. S., 1981; Rapp and Todaro, 1980; and Stahl, et al., 1977). Such a sequence labelling of the cellular gene should then allow its identification by molecular cloning. To this end, an in vitro system was developed for the transformation of mouse epithelial cells by MuLV in conjunction with TPA. The target cells were MMCE Cl 7 (Rapp et al., 1979) a cell line that can be reversibly transformed by TPA. MMCE Cl 7 can be transformed at very low frequency by infection with ecotropic C3H/MuLV. When infection of MMCE cells was followed by growth in TPA-substituted medium (100 ug/ml) and the cells were subsequently tested for growth in soft agar in the absence of TPA, a dramatic increase in the frequency of transformation was observed (from 0-4 colonies/10^5 cells to 600-800 colonies/10^5 cells). Cells transformed by MuLV and TPA retained a high plating efficiency in soft agar and generally did not produce virus particles. This basic protocol was also applied to rat fibroblast cells and while background growth of rat cells in agar + TPA was higher than that observed with the MMCE cells (50-70 colonies/10^5 cells) a 2-5 fold increase in the number of agar colonies was observed after infection of the rat cells with ecotropic C3H/MuLV and subsequent treatment with TPA.

4. Analysis of genes involved in chemical transformation of C3H/10T1/2 and MMCE cells in vitro. Two cell systems were used for chemical transformation in vitro, the fibroblast cell line C3H/10T1/2 and the epithelial mouse cell line MMCE. Carcinogens used included MCA (methylcholanthrene), ENU (ethylnitrosourea and ENU plus the promoter TPA. Transformed cells were cloned through soft

agar before further analysis. The DNAs from several of these clones have been tested by others for transforming activity (Shih et al., PNAS 76, 5714, 1979), Wigler, unpublished data) and were found to be positive. From these experiments it appeared that in these cells transformation was due to the action of presumably a single gene. We had tested previously whether such a gene may be an endogenous type C virus or a virus-cell recombinant by superinfection rescue and BUdR activation experiments. However, neither competent nor defective transforming viral genomes were inducable from these cells (Rapp et al., Virology 65: 392, 1975). Recently these experiments were extended as follows: 1. Transforming viral genomes were isolated from chemically transformed cells chronically infected with endogenous MuLV. We have molecularly cloned these genomes and examined their structure and biological activity. 2. Transformed cells were then examined for expression of these new oncogenic viral genomes as well as for a series of known type C virus coded tumor genes (Bister, Rapp, and Duesberg, unpublished data).

Significance to Biomedical Research and the Program of the Institute:

The overall goal of this work is to characterize cellular genes that are associated with the induction of cancer as it occurs spontaneously or after induction with carcinogens. Considering the fact that carcinomas are especially prevalent in humans, we have recently put special emphasis on transformation of epithelial cells. A family of cell-derived transforming genes isolated as part of type-C viruses might provide the tools necessary for both an understanding of mechanisms of transformation, and the development of strategies to neutralize their action.

Proposed Course:

Recent evidence from transfection experiments with chromosomal DNA has shown (Shih et al., Proc. Natl. Acad. Sci. USA 76: 5714, 1979) that chemical transformation of cells may be achieved by the activation of single tumor genes. These genes may be altered as a consequence of carcinogen treatment or may represent derepressed normal genes. Those chemically transformed cells that were efficient donors of transforming DNA will be tested for their effectiveness as target cells for the generation of new transforming retroviruses. It appears that both DNA transfection and type-C virus mediated transduction will lend themselves to the study of genetic changes related to chemical carcinogens.

Publications:

Keski-Oja, J., De Larco, J.E., Rapp, U.R., and Todaro, G.J.: Murine sarcoma growth factors affect the growth and morphology of cultured mouse epithelial cells. J. Cell. Physiol. 104: 41-46, 1980.

Keski-Oja, J., Heine, U.I., Wetzel, B., and Rapp, U.R.: Epidermal growth factor-induced alterations in proliferating mouse epithelial cells. Exp. Cell Res. 128: 279-290, 1980.

Rapp, U.R., and Todaro, G.J.: Generation of oncogenic mouse type C viruses: In Vitro selection of carcinoma-inducing variants. Proc. Natl. Acad. Sci. USA 77: 624-628, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05153-02 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of the Biological Activity of Cloned Recombinant MSV DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Donald G. Blair	Expert	LVC	NCI
OTHER:	Peter J. Fischinger	Medical Director	LVC	NCI
	Thomas G. Wood	Senior Staff Fellow	LMV	NCI
	William L. McClements	Senior Staff Fellow	LMV	NCI
	Marianne K. Oskarsson	Chemist	LMV	NCI
	William G. Robey	Research Chemist (Biochem.)	LVC	NCI
	George F. Vande Woude	Supervisory Research Chemist	LMC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Virus Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.675	0.7	0.975

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Moloney murine sarcoma virus (MSV)-related normal cell sequence c-mos, cloned from Balb/c mouse cells, is identical to the MSV-specific transforming sequence v-mos, but is unable to transform cells in DNA transfection assays. Mos is the specific transforming sequence carried by the Moloney MSV. V-mos refers to that sequence found in the viral genome, while c-mos is its cellular homology found in normal mouse cells. The transforming potential of c-mos was activated by linking it to the MSV long terminal repeat (LTR) sequence derived from a recombinant DNA clone containing the whole MSV provirus. The LTR is a 600 base pair sequence which contains presumptive RNA control sequences and which is repeated at both the 3' and 5' termini of the provirus. We are characterizing the nature of mos-containing RNA expressed in these transformed cells. We are attempting to activate the expression of other transforming sequences by linking the viral LTR to these sequences and measuring their ability to transform normal mouse cells.

Project Description

Objectives:

To define the functions of specific portions of the Moloney murine sarcoma virus (MSV) genome in MSV transformation and to identify specific genetic sequences necessary to activate the transformation potential of normal cell sequences of mouse and human origin.

Methods Employed:

Standard tissue culture techniques for the growth and assay of leukemia and sarcoma viruses; Ca^{++}-PO_4-DNA transfection of tissue culture cells; electrophoresis and Northern and Southern blot hybridization analysis of cell DNA and poly A selected cell and viral RNA.

Major Findings:

1. A normal mouse genetic sequence can be activated to exhibit transforming potential. The normal mouse cell DNA homologue of the MSV transforming sequence (c-mos) was initially shown to be capable of transforming cells in culture with high efficiency when linked to sequences derived from the 5' end of MSV, including a single copy of the MSV long terminal repeat (LTR). Additional clones were constructed which contained only the LTR linked to c-mos and a variable amount of intervening normal mouse sequences. These clones were able to transform cells with efficiencies identical to those determined earlier for MSV subgenomic fragments which contained mos and a single copy of the viral LTR. Fragments of cloned mouse DNA containing c-mos, but lacking LTR sequences, lacked the ability to induce transformation. Cells transformed by LTR-c-mos DNA expressed several new discrete RNA species which contained sequences homologous to mos, the LTR and the pBR322 vector in which these sequences were cloned. The viral LTR is known to contain presumptive RNA synthesis control elements. These results demonstrate that the oncogenic potential of a normal mouse sequence, c-mos could be activated by the addition of a specific viral-derived sequence. They further suggest that this activation involves the promotion of RNA expression of previously quiescent genetic sequences, and that this effect is nonselective in that prokaryotic pBR322 plasmid sequences are also expressed.

2. A subgenomic fragment of MSV can be rescued by MuLV superinfection. Attempts were made to recover infectious MSV from cells transfected by subgenomic fragments of MSV proviral DNA. Low levels of infectious MSV could be recovered from cells transfected by fragments containing the 5' portion of MSV, including the LTR and mos, and superinfected with MuLV. The rescued MSV is able to replicate to high titer after several cycles of viral growth. Analysis of viral RNA from the first cycle of virus rescue indicates the virus containing a novel large RNA species which contains both mos and pBR322 cloning vector sequences. This suggests that under appropriate conditions RNA tumor viruses may be capable of packaging nonviral information linked to viral sequences.

3. Activation of mos transformation by cotransfection can regenerate infectious MSV. Previous results had indicated that cotransfection of

separate fragments containing the viral LTR and a poorly transforming MSV proviral DNA fragment, which contains mos but lacks LTR sequences, results in an 100-300 fold enhancement in the efficiency of transformation. We have extended this result by showing that a clone containing the LTR and additional sequences coding for the MSV-specific p60 polyprotein will also enhance the transforming efficiency of mos in cotransfection. The resulting transformed cells also exhibit p60 expression, as measured by immunofluorescence. Infectious MSV can be recovered from a subset of these transformed clones following MuLV superinfection. The frequency of rescue is enhanced if the mixture of DNA fragments is first treated with polynucleotide ligase prior to transfection.

Significance to Biomedical Research and the Program of the Institute:

The identification of specific sequences which will "turn on" inactive sequences to an active transforming state provides a means for identification, isolation and characterization of such sequences. Thus by linking cloned LTR sequences to randomly sheared or fragmented normal cell DNA, it should be possible to occassionally position these sequences adjacent to genes with transforming potential. These transforming sequences, which are usually not transcribed and are only activated by either spontaneous or chemically induced carcinogenesis, would presumably be activated by the LTR. These activated transforming sequences could be detected by their ability to transform cells following DNA transfection, and their isolation and subsequent cloning would make use of their linkage to LTR and MSV-gag coding sequences, which can be monitored biochemically.

The ability to process, package and amplify such sequences as infectious virions could greatly extend our ability to detect such sequences. It would allow the utilization of the viral RNA control and splicing signals and allow the packaging and isolation of sequences which may be too large to isolate by conventional cloning techniques. For example, a 20 kb DNA sequence may contain 6 kb of sequences coding for a transforming protein. The entire 20 kb fragment would be difficult to clone by conventional techniques, but the spliced 6 kb piece, if packaged into a virus particle under the influence of a viral promoter and splicing signals, could be readily manipulated, cloned, and analyzed. Thus this type of cloning vector should facilitate the identification, isolation and characterization of a wider variety of transforming sequences. We are currently attempting to isolate such sequences from normal human DNA.

Proposed Course:

Analysis of mos-containing RNA of transfected cells will be continued to further dissect the mechanism and role of specific LTR sequences in the activation. Further attempts will be made to define an optimal method for the activation of unexpressed sequences using the viral LTR. The use of vectors containing the LTR associated with different amounts of MSV-derived sequences coding for gag-containing polyproteins will be extended in order to provide a second marker for screening and selecting successful LTR activations of transforming genes. The structure and properties of MSV rescued from cells transformed with subgenomic fragments will be analyzed to ascertain the mechanism of rescue and to establish the presence of additional transduced sequences. Attempts will continue to activate transforming sequences present in normal human DNA and human tumor cell lines and tissues.

Publications:

Blair, D.G., McClements, W.L., Oskarsson, M.K., Fischinger, P.J., and Vande Woude, G.F.: The biological activity of cloned Moloney sarcoma virus DNA-Terminally redundant sequences may enhance transformation efficiency. Proc. Natl. Acad. Sci. USA 77: 3504-3508, 1980.

Vande Woude, G.F., Oskarsson, M.K., McClements, W.L., Enquist, L.W., Blair, D.G., Fischinger, P.J., Maizel, J.V., and Sullivan, M.: Characterization of integrated Moloney sarcoma proviruses and flanking host sequences cloned in bacteriophage lambda. Cold Spring Harbor Symp. Quant. Biol. 44: 735-746, 1980.

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McClements, W.L., Dhar, R., Blair, D.G., Enquist, L., Oskarsson, M.K., and Vande Woude, G.F.: The long terminal repeat of Moloney sarcoma provirus. Cold Spring Harbor Symp. Quant. Biol. 45, in press.

Blair, D.G., Oskarsson, M.K., McClements, W.L., and Vande Woude, G.F.: The long terminal repeat of Moloney sarcoma provirus enhances transformation. In Neth, R., Gallo, R.C., Graaf, T., Mannweiler, K., and Winkler, K. (Eds.): Haematology and Blood Transfusion, Vol. 26, Modern Trends in Human Leukemia, Berlin, Heidelberg, Springer-Verlag, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05155-02 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role and Control of Recombinant Viruses in Mammalian Leukemia Model Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Peter J. Fischinger	Medical Director	LVC	NCI
OTHERS:	Donald G. Blair	Expert	LVC	NCI
	William G. Robey	Research Chemist (Biochem.)	LVC	NCI

COOPERATING UNITS (if any) D.P. Bolognesi, Duke University Medical Center, Durham, NC; J. Ihle, Litton Bionetics, Inc. (FCRC), Frederick, MD; J.-P. Levy, Groupe de Recherches Inserm, Paris, France; W. Schafer, Max-Planck Institut, Tubingen, Germany; L. Chieco-Bianchi, University of Padova, Italy

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Virus Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	0.7	1.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Novel murine recombinant murine leukemia viruses (MuLV's) were isolated from naturally occurring and virus-induced murine leukemias. Selective host systems were derived. De novo recombination was detected in ecotropic MuLV-induced leukemia. Recombinant envelope glycoprotein (gp)70 molecules were found in virus-free lymphomas. New models of leukemogenesis were developed. Seroimmunoprophylaxis with anti-MuLV gp70 IgG was successful in AKR mice. Virus release was eliminated. Breeding of protected AKR mice yielded sequential generations of virus-negative AKR mice.

Project Description

Objectives:

To understand the role of recombinant murine leukemia virus (MuLV) in virus-induced as well as virus-free mouse leukemia. To define the permuted envelope glycoprotein (gp)70 region on the molecular level and to clone molecularly various regions of the genome. To postulate a new hypothesis of leukemogenesis in which recombinant MuLV's indefinitely expand a receptor-defined subpopulation of lymphoid cells. To examine the natural history of disease in high and low incidence strains of mice and to see how effective interventive measures could be. To continue the successful seroimmunotherapy program of AKR leukemia and to develop virus- and disease-negative AKR mice.

Methods Employed:

Tissue culture, including propagation and derivation of normal and tumor cultures. Standard virological assays such as focus assay for both transforming and nontransforming oncornaviruses, soft agar assays, isotopic precursor incorporation, isolation and purification of viruses, density gradient centrifugation. Isolation and assay of protein and nucleic acid components of viruses and cells including reverse transcriptase assays, nucleic acid hybridization, restriction enzyme analyses, and cloning of DNA fragments.

Major Findings:

1. Isolation of recombinant MuLV's from murine leukemias. All mouse leukemias examined, which were induced by standard strains of ecotropic MuLV, were found to contain recombinant (RM) MuLV with specific xenotropic MuLV substitutions in the env gene gp70 product. RM-MuLV's were generally not detectable because these genomes were masked with the ecotropic MuLV coats. Several new oncogenic strains of RM-MuLV were isolated from Moloney MuLV stocks which did not contain the usual xenotropic peptide. Novel RM-MuLV's were also obtained from Graffi MuLV stocks and also from normal C57Bl mouse cells transformed by MSV. An unusual RM-MuLV isolation took place in the case of virus-positive SJL mice. Although these mice have high titers of ecotropic MuLV throughout life, the lymphomas that develop have little or no ecotropic MuLV, but essentially all tumors have RM-MuLV. This RM-MuLV, appearing under natural conditions, seems to be most related to Rauscher MuLV.

2. Growth restriction of recombinant MuLV's. Because of genomic masking and lack of a cell system selective for xenotropic MuLV and against RM-MuLV, a number of cells were examined for their ability to discriminate against RM-MuLV in order to study the xenotropic MuLV precursor to recombination. Of several cell species tested, diploid goat cells were found to be completely permissive for xenotropic MuLV and completely exclusive for RM-MuLV. One can now easily isolate xenotropic MuLV from mixed MuLV stocks.

3. Role of recombinant MuLV's in murine leukemia. The necessity for RM-MuLV in mouse leukemogenesis was examined by asking first whether genomically pure

ecotropic MuLV could give rise to RM-MuLV during leukemogenesis. A genetically cloned strain of Moloney MuLV never gave rise to RM-MuLV in tissue culture, but was able to induce leukemias in Balb/c mice, which all contain de novo generated RM-MuLV. Another approach was directed at an exceptional case: the virus-positive mouse which never becomes leukemic. A prototype strain CBA/N with sex-linked immunological defect becomes highly viremic after Moloney MuLV infection but no leukemia develops. These mice were found to have RM-MuLV in titers similar to leukemic Balb/c mice. In this case, perhaps the failure of immunological cell response may reflect a lack of appropriate target cells for the virus.

4. Recombinant MuLV-related products in virus-free leukemias. Virus-free leukemias were examined for discrete virus products. A strain of X-ray-induced, Thy 1-positive, immature mouse thymoma cells was isolated, which was negative by radioimmune assays for any MuLV protein. However, testing with an antiserum, which was class reactive with RM-MuLV, detected a gp70 on the cell surface in 5-10% of cells. On cloning, all cells contained the capacity to express this gp70 as seen also by cytotoxicity protein A binding and radioimmune precipitation tests. These cells excluded RM-MuLV's but not ecotropic MuLV's presumably by interference. Peptide maps of the isolated molecule showed greatest relationship to RM-MuLV gp70. Analogous gp70's were detected in other virus-free mouse leukemia cells from different strains. We postulated that the presence of this gp70 together with a receptor could serve as a constant autostimulatory blastogenesis signal which causes the uncontrolled lymphoblastic proliferation in the absence of leukemogenic MuLV's.

5. Seroimmunoprophylaxis of AKR mouse leukemia. Extensive seroprophylaxis experiments were continued on disease modification of AKR mice. Goat IgG, highly reactive with type, group and interspecies specific determinants of Friend MuLV gp70 was used to treat AKR mothers and baby mice. If this was performed during the critical first three days after birth ("window"), the long term survival of the treated group was twice that of the control AKR group, i.e. two years vs. one year. Later treatment times of babies, or treatment of only mothers was much less effective. The average long term survivors had much less virus or AKR p12 antigen, and mounted an active, type specific neutralizing and precipitating immune response against the AKR gp70. In some mice, the ideal situation was as follows: no virus of any kind, no antigens, and very high precipitating and AKR neutralizing titers. These mice did not die of malignant disease. Breeding of long term survivors has resulted in offspring which are being tested. Long term survival of these mice has now been seen into the fourth generation without further treatment of offspring. Reexamination of viral events after treatment of baby mice with immune IgG were examined in vitro by infectious cell center experiments (ICC). Shortly after birth, there was an intense 100-1000-fold burst of virus activity in some organs, but ICC's in the thymus were initially low. This was only ecotropic MuLV and no RM-MuLV was found early. The role of Fv-1 genes was examined in F_1 crosses. Treatment with immune IgG completely suppressed this MuLV release. Immunologically virus antigen positive cells were eliminated as well.

Significance to Biomedical Research and the Program of the Institute:

The role of RM-MuLV is being more clearly defined in mouse leukemia by examining the natural history of virus presence in many disease variants, as well as establishing postulates of casuality by genetically pure virus isolates. The exceptional RM-MuLV positive strains which do not develop leukemia will define the role of the participating cell(s). Of particular value may be the conceptual transition from known and well defined viral mouse leukemogenesis to nonviral yet effective physical induction of leukemia with the link of a viral or virus-like surface molecule. The autostimulatory, cycling blastogenesis which appears to work in AKR viral lymphoid leukemia may be of real significance if this concept also holds true in other nonviral leukemia models. Specific attention could then be focused on immunological methods employing a line of nonproducing X-ray-induced thymoma cells (NIXT). Antibody to gp70 found on NIXT cell surface will be used to see whether nonviral disease modification could result. The successful seroimmunoprophylaxis collaborative program represents a functional, and holistic approach to the treatment of an overwhelming viral disease. As envisioned, the model system will continue to be defined in all its components, and specific additional inputs, both protective and detrimental, could be evaluated on a well worked out background. These may involve further cellular immunotherapy as well as accepted additional chemotherapeutic regimens.

Proposed Course:

The generation of RM-MuLV will be examined in more detail. This will involve a clear definition of the recombined env gene site on the molecular level using both protein and nucleic acid technology including recombinant DNA techniques. The structural anatomy of this site will be analyzed relative to the ecotropic MuLV flanks and especially the 5' long terminal repeats. The unusual RM-MuLV isolates will be of particular interest as well as RM-MuLV from nonleukemic strains of mice. Further studies will be performed on other virus negative mouse lymphomas to determine the type of gp70 on cell surface, as well as the potential responsiveness of host to such isolated gp70's. Because at this time it is not clear which xenotropic virus sequences contribute to RM-MuLV, we will examine with our new selective cell systems the xenotropic MuLV's in mixed MuLV stocks. The seroimmunoprophylaxis experiments will be continued in a major assessment of the role of ecotropic MuLV and RM-MuLV especially relative to the preleukemic period. The nature of the initial virus target cell which appears not to be a T cell will be examined. Breeding experiments of immune IgG-treated, virus-negative adults will continue to see whether a virus-negative but genetically unmodified AKR substrain could be developed by breaking the cycle of virus release and select immune unresponsiveness. Such systems could be useful for assessing perturbating environmental and physicochemical contributions which could distabilize the protected system.

Publications:

Fischinger, P.J., Ihle, J.N., Levy, J.P., Bolognesi, D.P., Elder, J. and Schafer, W.: Recombinant murine leukemia viruses and protective factors in leukemogenesis. In Essex, M., Todaro, G., and zur Hausen, H. (Eds.):

Viruses in Naturally Occurring Cancer. New York, Cold Spring Harbor Press, 1981, Vol. 7, pp. 989-1003.

Fischinger, P.J., Ihle, J.N., Thiel, H.-J., Bolognesi, D.P., and Elder, J.: Presence of recombinant murine leukemia virus glycoprotein on the surface of X-ray induced, virus negative Swiss mouse thymoma cells. Proc. Natl. Acad. Sci. USA 78: 1920-1924, 1981.

De Rossi, A., D'Andrea, E., Colombatti, A., Fischinger, P.J., and Chieco-Bianchi, L.: Studies on spontaneous lymphomas in SJL/J (v+) mice: ecotropic and dualtropic virus expression in normal and lymphomatous tissues. J. Natl. Cancer Inst, in press.

Schwarz, H., Ihle, J.N., Wecker, E., Fischinger, P.J., Thiel, H.-J., Bolognesi, D.P., and Schafer, W.: Properties of mouse leukemia viruses. XVII. Factors required for successful treatment of spontaneous AKR-leukemia by antibodies against gp71. Virology, in press.

Fischinger, P.J., Thiel, H.J., Blevins, C.S., and Dunlop, N.M.: Selective host-range restriction of goat cells for recombinant murine leukemia virus and feline leukemia virus type A. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05171-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Growth Factors Produced by Human Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hans Marquardt	Visiting Scientist	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	Joseph E. De Larco	Research Chemist	LVC	NCI
	Daniel R. Twardzik	Research Chemist	LVC	NCI
	Stephen A. Sherwin	Clinical Associate	BRMP	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project seeks to purify and characterize the major classes of growth factors produced and released by transformed cells. This includes chemical and biological comparisons with known hormones like insulin-like growth factor (IGF) and epidermal growth factor (EGF). A low molecular weight polypeptide with multiplication stimulating activity (MSA) was isolated from serum-free media conditioned by a clone of normal Buffalo rat liver cells. MSA is a single-chain polypeptide of 67 residues, with a calculated molecular weight of 7,484, and displays 93% homology with the functionally related human IGF-II. A human metastatic melanoma cell line (A2058) produces a transforming growth factor (TGF). TGF is functionally related to EGF and interacts with the same receptors on cultured cells. TGF, however, produces a profound phenotypic alteration on susceptible cells and confers the ability to behave as transformed cells. EGF, in contrast, does not produce large, progressively growing cell colonies in soft agar. TGF was isolated from serum-free media conditioned by A2058 cells. A comparison was made with human EGF (hEGF) isolated and purified from human urine.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To purify and characterize the major classes of growth factors released by transformed human cells in order to chemically and biologically compare the isolated polypeptides with known growth factors, such as insulin-like growth factor (IGF) and epidermal growth factor (EGF), and to determine their role in malignant transformation.

Methods Employed:

Biochemical and biological laboratory techniques were developed or adapted to isolate and purify growth factors from serum-free supernatants of cultured normal or tumor cells. Various standard techniques were used to characterize and compare the purified growth factors on the level of the primary structure. Antibodies were prepared and radioimmunoassays were developed for the isolated growth factors.

Major Findings:

1. Characterization of a multiplication stimulating activity-like polypeptide produced by a human fibrosarcoma cell line. This laboratory recently presented evidence that a line of human fibrosarcoma cells (8387) produces a growth factor which is functionally related to multiplication stimulating activity (MSA). MSA is a growth factor which was first described as a product of a normal Buffalo rat liver (BRL) cell line grown in serum-free medium. The rationale of these studies was to isolate the MSA-like growth factor from the supernatants of the 8387 cell line, and then to chemically and biologically compare this polypeptide hormone with MSA. The functional relationship of MSA to the insulin-like growth factors (IGF) has been demonstrated. Amino acid sequence data, however, were not available.

2. Purification and primary structure of a polypeptide with multiplication stimulating activity. The purification of MSA was achieved by gel-permeation chromatography of the acid-soluble growth-promoting activity on Bio-Gel P-10 in 1 M acetic acid and followed by reversed-phase high-pressure liquid chromatography (rph HPLC) on μBondapak C_{18} support using a linear gradient of 0.05% trifluoroacetic acid in acetonitrile. The primary structure of MSA has been determined. MSA is a single-chain polypeptide of 67 residues, with a calculated molecular weight of 7,484, and displays 93% homology with human IGF-II. A tentative primary structure for MSA, deduced from microsequence analysis data of unmodified MSA and tryptic peptides and from results of carboxypeptidase digestion of selected tryptic peptides and MSA, is:

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1    5    10    15    20    25    30    35

A-Y-R-P-S-E-T-L-C-G-G-E-L-V-D-T-L-Q-F-V-C-S-D-R-G-F-Y-F-S-R-P-S-G-R-A-

      40    45    50    55    60    65

N-R-R-S-R-G-I-V-E-E-C-C-F-R-S-C-D-L-A-L-L-E-T-Y-C-A-T-P-A-K-S-E
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A comparison between the sequences of rat MSA and human IGF-II reveals only five amino acid substitutions. Based on the extensive amino acid sequence homology, the term rat IGF-II was proposed for this newly isolated polypeptide.

3. Isolation and purification of a transforming growth factor produced by a human metastatic melanoma cell line. This laboratory has been actively involved in the identification and characterization of biologically active peptides, especially those of human tumor cell lines. A family of transforming growth factors (TGF) that are related, but distinct from EGF have been recently described. TGF is able to produce a rapid, readily detectable morphologic alteration of susceptible cells and confers the ability to behave as transformed cells. EGF, in contrast, has only a slight effect on monolayer cultures. Both EGF and TGF bind to the EGF membrane receptor protein.

To further characterize the TGF produced by A2058 cells, serum-free conditioned media were collected, concentrated, and partially purified by acid dialysis and centrifugation. The final purification of TGF was achieved by gel permeation chromatography on Bio-gel P-10 in 1 M acetic acid, followed by rp HPLC on Bondapak C_{18} support using a linear gradient of 0.05% trifluoroacetic acid in acetonitrile, and rechromatography on Bondapak C_{18} support using a linear gradient of 0.04% trifluoroacetic acid in 1-propanol. Individual fractions were tested for protein, ability to stimulate NRK cells to form colonies in soft agar, and ability to compete with mouse ^{125}I-labeled EGF on formalin-fixed A431 cells (human epidermoid carcinoma cells have an exceptionally high number of EGF receptors). The majority of the protein eluted in the exclusion volume of the P-10 column. A major peak of biological activity was found in the included volume, with an M_r=6,800. The P-10-pool was reconstituted in 0.05% trifluoroacetic acid in water, and then chromatographed on a μBondapak C_{18} column. The EGF-competing activity and the activity to stimulate cells to form colonies in soft agar co-eluted at 24% acetonitrile. Fractions containing TGF were pooled, lyophilized and rechromatographed on the same column support, using a linear gradient of 0.04% trifluoroacetic acid in 1-proponal for the final purification step of TGF. In the solvent system used, TGF was eluted at 13.5% propanol and was effectively separated from the main contaminating polypeptides. The recovery of TGF as a single UV-absorbing peptide peak was 90% of the starting material. The yield of purified TGF was 1 μg per 75 liters of A2058-conditioned medium. The soft agar growth stimulating activity and the EGF-competing activity eluted in a single fraction, corresponding to a UV-absorbing homogeneous peptide peak.

The biological properties of highly purified TGF were compared with human EGF (hEGF), isolated from midterm pregnancy urine, in the radioreceptor assay. Both TGF and EGF competed with ^{125}I-labeled hEGF for the EGF receptor sites of A431 cells. The slopes of the competition curves were similar, and TGF was on a weight basis as active as EGF. The soft agar growth stimulation assay was as sensitive as the EGF-competition assay. At a concentration of 1 ng TGF/ml, which gives a 50% reduction in ^{125}I-EGF binding, there was a readily detectable response in the soft agar assay (>50 large colonies per 10^4 cells, the control cultures showed no colonies per 10^6 cells).

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Significance to Biomedical Research and the Program of the Institute:

The successful isolation of a tumor specific growth factor has now made possible the elucidation of the primary structure in order to compare its structural features with hEGF. Preparation of monoclonal antibodies and development of radioimmunoassays will provide a diagnostic tool for screening human tumor tissues for TGF-like material to study the early onset of disease for preventative treatment.

Proposed Course:

The continuation of this work includes the purification of these growth factors in preparation for sequencing. Production of sufficient quantities will permit the study of the specific mechanism of action as well as its genetic relationship to the control of transformation, both genetically and phenotypically. Plans include the use of recombinant DNA techniques to prepare test quantities of these substances.

Publications:

Marquardt, H., Wilson, G.L., and Todaro, G.J.: Isolation and characterization of a multiplication-stimulating activity (MSA)-like polypeptide produced by a human fibrosarcoma cell line. J. Biol. Chem. 255: 9177-9181, 1980.

Marquardt, H., Todaro, G.J., Henderson, L.E., and Oroszlan, S.: Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures: Homology with human insulin-like growth factor II. J. Biol. Chem. in press.

Todaro, G.J., Marquardt, H., DeLarco, J.E., Fryling, C.M., Reynolds, F.H., and Stephenson, J.R.: Transforming growth factors produced by human tumor cells: Interaction with epidermal growth factor (EGF) membrane receptors. In Scott, W.A., Werner, R., and Schultz, J. (Eds.): Cellular Responses to Molecular Modulators. New York, Academic Press, in press.

Marquardt, H., and Todaro, G.J.: Isolation and characterization of a transforming growth factor produced by a human metastatic melanoma cell line. J. Biol. Chem., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05172-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Biosynthesis and Characterization of Transformation Specific Glycolipids and Glycoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mohinder S. Kang	Visiting Fellow	LVC	NCI
OTHER:	Leo A. Phillips	Research Microbiologist	LVC	NCI
	George J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)
Inderjit Singh, Johns Hopkins University, Baltimore, MD
Alan Elbein, Univ. Texas Health Science Center, San Antonio, TX

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.0	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibiotics which specifically inhibit synthesis of lipid-linked saccharides, intermediates of protein glycosylation, were studied. It was found that the antibiotic streptovirudin specifically inhibits glycosylation of viral glycoproteins, thereby blocking multiplication of vesicular stomatic virus. These results suggest that the carbohydrate moieties of viral glycoproteins are necessary for virion formation.

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Project Description

Objectives:

To study the role of glycolipids and glycoproteins in neoplastic transformation. For this purpose, specific inhibitors of glycolipid and glycoprotein synthesis will be employed to study the role of the carbohydrate portion of glycolipids and glycoproteins. Alterations in the glycolipid and glycoprotein synthesis of virally- and chemically-transformed cells, and changes in the activities of transformation-associated glycosyl transferases as tumor markers will be investigated. Binding sites for tumor promoters, e.g., phorbol esters, sarcoma growth factor and epidermal growth factor will be investigated. We are starting this new project to study and analyze the nature of these carbohydrate changes and their relationship to tumorigenicity.

Methods Employed:

Standard cell culture procedures, plaque assays and methods for primary mixed and isolated cell culture techniques; analytical and preparative ultracentrifugation; paper, column and thin layer chromatography; isotopic labelling; polyacrylamide gel electrophoresis; in vitro and in vivo testing of antibiotics; gas liquid chromatography and high performance liquid chromatography.

Major Findings:

1. Inhibition of glycoprotein biosynthesis by antibiotics. Cell surface carbohydrates have been shown to play a role in cell adhesion, secretion and a number of intracellular recognition processes which are altered during malignant transformation. Unfortunately, there have been few instances in which these carbohydrate changes have been analyzed for their relationship to, or effect on, tumorigenicity. We are starting this new project to study and analyze the nature of these carbohydrate changes and their relationship to tumorigenicity. Several compounds such as D-glucosamine, 2-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose are known to be the potent inhibitors of multiplication of enveloped viruses. This inhibition was shown to be caused by blocking the glycosylation of viral glycoproteins. However, since these compounds affect cellular processes other than glycosylation, the data obtained do not allow unequivocal interpretations. We have tested several antibiotics such as bacitracin, amphomycin, showdomycin, antibiotic 24010 and streptovirudin. These antibiotics in vitro have shown to be inhibitors of synthesis of lipid-linked saccharides, the intermediators of protein glycosylation. The role of these inhibitors in vivo has not yet been elucidated. These inhibitors can be useful in trying to understand the function of the carbohydrate portion of glycoproteins. We have used streptovirudin to study the role of glycosylation of viral glycoproteins of vesicular stomatitis virus (VSV). These results clearly demonstrate that the carbohydrate moiety of viral glycoproteins in VSV is necessary for virion formation.

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Significance to Biomedical Research and the Program of the Institute:

The changes in the carbohydrate portion of glycolipids, glycoproteins and certain glycosyl transferases associated with tumorigenicity in cell culture should help in a better understanding of the complicated process of carcinogenesis in humans, aid in early diagnosis of tumors, and possibly give opportunities to intervene in the carcinogenesis process or selectively kill the cancer cells.

Proposed Course:

Most of these investigations will be carried out in cultures of brain cells. The brain has proved to be a tissue of interest because of its high level of phorbol ester binding activity, high content of glycolipids and similarity of pathways for synthesis of glycolipids and glycoproteins to that of other organs. The mechanism of action of known inhibitors of protein glycosylation on retrovirus assembly and replication should help to analyze the role of the carbohydrate portion of glycoprotein in virion formation. Knowledge of the effects of tumor promotors, and chemical carcinogens on the changes in glycosyl-transferases, glycolipids and glycoproteins, and characterization of binding sites for binding of tumor promotors should help in understanding the mechanism of carcinogenesis.

Publications:

Elbein, A.D., Heifetz, A., Kang, M.S., Spencer, J., and Keenan, R.W.: Inhibition of lipid-linked saccharide formation by antibiotics. In Marshall, J.J. (Ed.): Mechanisms of Saccharide Polymerization and Depolymerization. New York, Academic Press, Inc., 1980, pp. 1-20.

Kang, M.S., Park, J.J., Singh, I., and Phillips, L.A.: Streptovirudin inhibits glycosylation and multiplication of vesicular stomatitis virus. Biochem. Biophys. Res. Commun. 99: 422-428, 1981.

Phillips, L.A., Kang, M.S., and Hollis, V.W. Jr.: Complementary DNA copies of leukemia and sarcoma virus RNA contain sequences of deoxycytidylate and deoxyguanylate. In Nieburgs, H.E. (Ed.): Cancer Detection and Prevention. New York, Marcel Dekker, Inc., in press.

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BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05173-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Hormone and Growth Factor Requirements of Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Angie Rizzino	Expert	LVC	NCI
OTHER:	Nancy H. Colburn	Expert	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.80	0.60	0.20

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The hormone and growth factor requirements of mammalian cells have been observed by several laboratories to change after viral transformation. This project sought to determine whether transformation induced by tumor promoters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), would also effect the hormone and/or growth factor requirements of cells. Toward this goal a hormone and growth factor supplemented defined medium was developed for an epithelial cell line (JB-6 CLONE 41), which undergoes transformation after exposure to TPA. In place of serum, the defined medium contains fibronectin, insulin, transferrin and epidermal growth factor (EGF). In this defined medium the cells can be grown indefinitely, but if EGF is omitted the cells exhibit little or no proliferation. Using this defined medium as a starting point, it was determined that a transformed cell line isolated from JB-6 Clone 41 will proliferate in defined media lacking EGF. Thus, these studies indicate that transformation by tumor promoters can lead to altered growth factor requirements.

PHS-6040
(Rev. 2-81)

BEST DOCUMENT AVAILABLE

Project Description

Objectives:

Current evidence strongly supports the argument that hormones and growth factors play a central role in the control of cell proliferation. This project has begun to examine the changes in the requirements for hormones and growth factors that occur when cells become transformed by tumor promoters. Specifically the requirements of non-transformed epithelial cells are compared to their transformed counterparts. The first step toward this objective is achieved by developing hormone and growth factor supplemented defined media able to support the growth of both cell types.

Methods Employed:

During the past five years it has been established that the serum requirement for cell growth in vitro can be partially or completely satisfied by substituting purified attachment factors, hormones and growth factors. This realization led to the development of hormone and growth factor supplemented defined media for a wide range of anchorage dependent cell lines (Rizzino et al., Nutritional Reviews 37: 369-378, 1979). Such media are most readily developed by first providing the cells with an appropriate substrate for attachment. In many cases tissue culture plastic is not a suitable substrate and an attachment factor is required (e.g. fibronectin). Once this requirement has been satisfied it is generally possible to stimulate and maintain cell proliferation by addition of appropriate hormones and growth factors. These must be determined for each cell line since different cell types required different combination of hormones and growth factors. Thanks to the work with defined media during the past five years, the task of determining the required factors has been simplified. Based on the current body of knowledge it is often possible to predict which factors are most likely to be required. In the case of the epithelial cells used in this study it was expected that epidermal growth factor (EGF), insulin, and transferrin would stimulate cell proliferation.

Major Findings:

1. Defined medium for JB-6 Clone 41. JB-6 Clone 41 are normally grown in medium containing 8% fetal calf serum. When the cells are transferred from serum-containing to serum-free media, the cells rapidly attach to the culture dish. Using this basic approach, defined media were developed for an epithelial cell line and a transformed cell line derived from it. JB-6 Clone 41 is an epithelial cell line that can be transformed by tumor promoters, such as 12-O-tetradecancyl-phorbol-13-acetate (TPA) (Colburn et al., "Carcinogenesis Vol. 1, Mechanisms of Tumor Production and Cocarcinogenesis", Raven Press 1978, pp 257-271). A transformed cell line, T^3, was isolated from JB-6 Clone 41 after exposure to TPA. A comparison of the hormone and growth factor requirements of these two cell lines has been made and is discussed below. However, during the next 48 hours nearly all of the cells detach and lyse. This problem was eliminated by coating the culture dishes with the attachment factor, fibronectin (FN). Under these conditions the cells exhibit little proliferation and eventually detach unless the required hormones and growth factors are also added. These were found to be insulin, transferrin (an iron transport protein) and EGF. In a defined medium containing the four factors, JB-6 Clone 41 can be grown and

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subcultured for numerous generations (at the time of this writing over 20 generations). Although each of the four factors is essential for prolonged growth in defined medium, EGF has the most effect. In the presence of FN, insulin and transferrin, net cell growth does not occur unless EGF is also present. These data demonstrate that EGF is a potent mitogen for JB-6 Clone 41 and is sufficient in the presence of FN, insulin, and transferrin to maintain the long-term growth of these cells in the absence of serum.

2. The growth of transformed cells in defined media. A transformed cell line T^3, has been derived from JB-6 Clone 41 after exposure to TPA (Colburn, unpublished results). The growth factor requirements of T^3 were found to differ from those of the parent cell line. While JB-6 Clone 41 shows a strict growth requirement for EGF in defined media, T^3 does not. Unlike JB-6 Clone 41, T^3 can be grown for at least three generations in defined medium containing only FN, insulin and transferrin. However, these cells do respond to EGF and this response appears to be density dependent. At low density EGF stimulates the growth of T^3 by over 50% but, at a 5-fold higher cell density, growth is stimulated less than 15%. These findings strongly suggest that one of the consequences of transformation by the tumor promoter TPA is a change in the requirement for EGF. Furthermore, the finding that the growth response of T^3 to EGF is density dependent suggests that T^3 may produce an EGF-like growth factor.

Significance to Biomedial Research and the Program of the Institute:

During the past five years, it has been established that viral transformation can lead to alterations in the growth factor requirements for cell proliferation. Although the mechanism(s) involved have not been established, it is probable that the transformed cells, in at least some cases, acquire the capacity to produce one or more of their required growth factors. This possibility has been supported by the work of De Larco and Todaro (PNAS, 75: 4001-4005, 1978) and may help explain the lack of normal growth controls exhibited by malignant cells. The overall goal of this work is to determine whether transformation by tumor promoters also results in modified growth factor requirements and to determine if these modifications are due to the production of growth factors by the transformed cells. In this study, epithelial cells were used in the hope of better understanding the most prevalent form of human cancers-carcinomas.

Proposed Course:

Recent evidence has shown that cells transformed by sarcoma viruses produce an EGF-like growth factor called sarcoma growth factor (SGF) which is largely, if not completely, responsible for the cells' transformed phenotype. Based on the finding that JB-6 Clone 41 becomes less responsive to EGF after transformation by a tumor promoter, it is important to determine whether the transformed cells also produce an EGF-like growth factor. This possibility will be tested by preparing conditioned media from T^3 and other transformed cell lines derived from JB-6 Clone 41. This task has been greatly simplified since a defined medium for the growth of T^3 is now available. The conditioned media will then be tested for ability to promote the growth of the untransformed parent cells in defined medium lacking EGF. If the cells are found to produce a growth factor it will be characterized and compared to EGF and SGF.

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Publications:

Darmon, M., Serrero, G., Rizzino, A., and Sato, G.: Isolation of myoblastic, fibro-adipogenic and fibroblastic clonal cell lines from a common precursor and study of their requirements for growth and differentiation. Exp. Cell Res. 132: 313-327, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05174-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Control of Differentiation by Extracellular Signals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Angie Rizzino	Expert	LVC	NCI
OTHER:	Joseph DeLarco	Research Chemist	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.60	0.40	0.20

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hormone supplemented defined media able to support the growth and differentiation of several embryonal carcinoma (EC) cell lines have been developed in order to identify extracellular signals that induce differentiation. Previously a defined medium (EM-3) containing fibronectin, insulin and transferrin was developed for the growth of the EC cell line F_9. In EM-3 F_9, cells undergo little or no differentiation unless an inducer such as retinoic acid is added. In direct contrast to these results at least two other EC cell lines spontaneously differentiate when transferred from serum containing media to defined media. The cells formed are similar in two respects to those formed when F_9 are exposed to retinoic acid: their morphology and the secretion of plasminogen activator. These results strongly suggest that a factor(s), present in serum, blocks the differentiation of some, but not other, EC cell lines. In related studies the growth requirements of F_9 lines and their differentiated cells were examined further. Epidermal growth factor and sarcoma growth factor were found to stimulate the growth of the differentiated cells but not that of the parent cells. Growth of F_9 at low density was found to require the addition of lipoproteins to EM-3.

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Project Description

Objectives:

Current evidence indicates that the extracellular environment affects differentiation. However, the mechanisms involved are essentially unknown. One of the major reasons for this has been the failure to identify the environmental signals involved. If the inducers could be identified, then their molecular modes of action could be examined and a better understanding of cellular differentiation would be achieved. During the past decade, it has become apparent that embryonal carcinoma cells (the stem cells of teratocarcinomas) represent an excellent model system for the study of cellular differentiation in general and early mammalian embryogenesis in particular. The primary objective of this work is to identify and study the signals (hormones, growth factors, etc.) that regulate the differentiation of embryonal carcinoma (EC) cells. Until recently the study of EC cells could only be conducted in serum-containing media. Due to the complexity and undefined nature of serum, it has been difficult to pinpoint the serum factors that affect differentiation. Clearly, it would be preferable to study EC cell differentiation in completely defined culture media selectively supplemented with required factors. The use of defined media would not only make it possible to determine which molecules are involved but would also permit a careful examination of their modes of action without the interference of extraneous serum factors.

Methods Employed:

Recently a hormone-supplemented defined medium was developed for the long-term growth of the EC cell line, F_9. The defined medium, referred to as EM-3, contains fibronectin, insulin and transferrin in the place of serum. When retinoic acid is added to EM-3, the F_9 cells irreversibly differentiate to cells that appear by several criteria, to be parietal endoderm. The main difference between the growth of F_9 cells in serum-containing medium and in EM-3 is the failure of F_9 cells to grow at low density in defined medium. The observation that their growth is density-dependent argues that the cells require a serum factor for growth at low density. Alternatively the cells may produce a factor(s) required for their own growth which becomes limiting at low density. If this is the case, then serum provides either the same factor(s) or another that can satisfy the requirement. The failure of F_9 cells to grow at low density in defined media provides a convenient assay for identifying molecules that support growth at low density. Using this assay, it was previously determined that F_9 cells in defined media condition their substrate. In the current study factors present in serum, as well as those produced by F_9, cells were examined for their ability to support growth at low density. These factors have been found to affect not only growth but also differentiation.

Major Findings:

1. Growth of F_9 embryonal carcinoma cells at low density in defined media.

F_9 EC cells can be grown at normal cell densities (10^4 cells per cm^2) in defined medium (EM-3). However, at a density 10- to 20-fold lower, many of the cells do not survive and cell proliferation is very limited. This problem can be overcome by the addition of purified serum lipoproteins to EM-3. Each of the three

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major classes of lipoproteins, high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) have been found to support continuous cell growth in defined media at the lower density. Although the function of the lipo-proteins is not clear, several lines of evidence argue that the lipoproteins are providing the cells with a source of lipids. Lipoproteins are known to play an important role in lipid metabolism and act as carriers for many molecules including both free and fatty acid-esterified cholesterol, various phospholipids (e.g. phosphatidyl choline) and triglycimides. To test the possibility that the lipoproteins may function by providing some of these molecules, the lipids of HDL were isolated and tested on their own. The results indicate that the HDL lipids significantly improve the growth of F_9 cells in defined media at low density but are not as effective as unfractionated HDL. This is not surprising since it is well established that lipoproteins are taken up by cells in order to efficiently acquire the lipids present in the lipoprotein complexes. Using a somewhat different approach, further evidence for the importance of lipids was obtained. Serum albumin, a carrier of fatty acids, was found to significantly improve the growth of F_9 cells at low density. In this case the effect of fatty acids is clear cut. Although bovine serum albumin (BSA) is very effective, fatty acid-free BSA is without effect unless fatty acids, such as oleic acid, are also added. In other studies, ethanolamine, an important precursor for several membrane lipids, was found to stimulate the growth of F_9 cells in EM-3 at low density. Taken together, these findings strongly suggest that various lipids are important supplements to the defined media for F_9 EC cells.

2. The growth of other embryonal carcinoma cells in defined media. The work with F_9 cells has been extended to other EC cell lines and very similar results were obtained with the EC cell line, PC-13. These cells also grow at high density in EM-3 but require lipoproteins or fatty acid free BSA plus oleic acid in order to grow at low density. In direct contrast to these results two other EC cell lines fail to grow in EM-3 or EM-3 supplemented with lipoproteins, even at high cell densities. Instead, when these cells are transferred from serum containing medium to EM-3 plus lipoproteins they spontaneously and irreversibly differentiate to cells that exhibit the morphology of parietal endoderm. Furthermore, the differentiated cells, like parietal endoderm and unlike EC cells, secrete plasminogen activator. It should be stressed that the survival of the differentiated cells in defined media requires the presence of lipoproteins. These results strongly contrast with the findings observed with F_9 and PC-13 cells, which for all intents and purposes, do not differentiate in defined or serum-containing media unless exposed to inducing agents, such as retinoic acid. Although the reason why these four EC cell lines behave differently is not entirely clear, the data strongly suggest that serum contains a factor(s) that blocks the differentiation of some but not other EC cell lines.

3. Requirements of the differentiated cells derived from EC cells. Recently, Rees et al., Nature 281: 309-311, (1980) have examined the growth factor requirements of PC-13 cells and their differentiated cells in serum-containing media. When PC-13 cells are induced by retinoic acid to differentiate there is a large increase in EGF receptors and the differentiated cells, unlike the parent EC cells, respond to epidermal growth factor (EGF) by increased cell proliferation. We have confirmed these observations and observed similar results with F_9 cells, which do not respond to EGF whereas their differentiated cells

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do. Furthermore, we have also observed that the differentiated cells of F_9 and PC-13 cells respond to the sarcoma growth factor (SGF). (This molecule is distinct from EGF but shares a number of its important properties, including binding to EGF receptors). The fact that the differentiated cells respond to both SGF and EGF, while F_9 and PC-13 cells do not, has raised the possibility that the EC cells produce an EGF-like growth factor and thus do not require or respond to exogenously added EGF or SGF. Preliminary studies support this possibility.

Significance to Biomedical Research and the Program of the Institute:

The major goal of this project is to identify extracellular signals (hormones etc.) that control differentiation. This work will prove to be important in two major areas. First, it will significantly broaden our understanding of cellular differentiation by identifying many of the inducers involved. This in turn will lead directly to the study of their molecular modes of action, making it possible to understand differentiation at the molecular level. As an additional bonus this work has revealed, and should continue to reveal, important differences between the differentiation of EC cells in serum-containing media and in defined media. The fact that such differences exist, indicates that work with EC cells in defined media adds a new and important dimension to the study of differentiation. Second, this work should eventually help to evaluate a new approach to cancer therapy. It has been argued (Pierce, In *Teratomas and Differentiation*, Sherman and Solter (eds.), Academic Press pp. 3-12 (1975) that the stem cells of certain tumor, (e.g., squamous cell carcinomas and neuroblastomas) have the capacity to differentiate in vivo to form harmless post-mitotic cell types. Under normal conditions the frequency of differentiation is usually low but it may be possible to retard or completely block the growth of certain tumors by inducing their stem cells to differentiate. However, before we can adequately evaluate such an approach we must determine how and to what extent the extracellular environment influences cellular differentiation.

Proposed Course:

Future studies will continue the work discussed above and will be focused on three main areas. First, it will be determined whether most EC cell lines undergo differentiation when transferred from serum-containing media to the defined medium discussed above. This will include further characterization of the cell type(s) formed when certain EC cell lines are transferred to defined medium. Second, it will be determined whether F_9 and other EC cells produce an EGF-like growth factor that affects the growth of EC cells and their differentiated cells. If they do, the factor will be isolated, characterized and compared to both EGF and SGF. Third, additional factors required for the clonal growth of F_9 and PC-13 EC cells in defined media will be identified, isolated and characterized.

Publications:

Rizzino, A., and Crowley, C.: Growth and differentiation of embryonal carcinoma cell line F in defined media. Proc. Natl. Acad. Sci. USA 77: 457-461, 1980.

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Rizzino, A., Terranova, V., Rohrbach, D., Crowley, C., and Rizzino, H.: The effects of laminin on the growth and differentiation of embryonal carcinoma cells in defined media. J. Supramol. Struct. 13: 243-253, 1980.

Darmon, M., Serrero, G., Rizzino, A., and Sato, G.: Isolation of myoblastic, fibro-adipogenic and fibroblastic clonal cell lines from a common precursor and study of their requirements for growth and differentiation. Exp. Cell Res. 132: 313-327, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05175-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Transforming Growth Factors: Expression During Embryogenesis and Detection in Human Urine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Daniel R. Twardzik	Research Chemist	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	Stephen A. Sherwin	Clinical Associate	BRMP	NCI
	Hans Marquardt	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.05	0.95	0.10

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse embryos contain transforming growth factors (TGFs) that, like those derived from tumor cells, compete with epidermal growth factor for binding to its specific receptor. Utilizing an acid/ethanol extraction technique previously described for the isolation of these factors from tumor cells, these activities have been demonstrated in embryos from six different strains of mice. Two major EGF-competing activities with estimated molecular weights of 10K and 17K have been partially purified from embryo extracts. Both activities support soft agar colony growth in contrast to mouse EGF which is also found, albeit a minor component, in the mouse embryo. TGFs have also been detected in human amniotic fluid and in urine collected from pregnant donors. Both a 17-20K and a 10K EGF competing activity which support soft agar colony growth can be demonstrated in acid/ethanol extracted urine preparations. In addition an 8K variant of EGF which competes for EGF receptors and supports soft agar growth is also found in all urine samples tested including normal donors. Similar TGF activities have also been detected in the urine of a patient with small cell carcinoma of the lung.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To develop a technique applicable to selectively extract and identify acid soluble polypeptides with phenotypic transforming activity from bulk embryonic tissues and other body fluids. Embryonic transforming growth factors (TGFs), which compete for epidermal growth factor (EGF) receptors and support soft agar colony activity, will be purified. Their biochemical and biological properties will be compared to the sarcoma growth factors (SGFs) identified and characterized from murine sarcoma virus (MuSV) transformed cells. The expression of these TGFs during different stages of embryogenesis and their detection under normal physiological conditions, pregnancy and the diseased state will be examined.

Methods Employed:

Hysterectomy-derived barrier-maintained mice were mated and age of gestation determined from coital plug formation and mean fetal length (mm). Embryos at different stages of development were surgically removed, and placenta and maternal membranes carefully dissected from the fetal mass. Freshly removed embryos were minced in an acidified ethanol buffer and acid soluble polypeptides precipitated with ethanol/ether. These acid/ethanol-extracted polypeptides were resolved by gel filtration into different molecular weights and tested for EGF-competing activity as well as for the ability to cause normal fibroblasts to form colonies in soft agar. Polypeptides which demonstrate these activities were further characterized utilizing high pressure liquid chromatography (HPLC) and compared to the elution properties of other known growth factors (i.e. SGF). The detection of similar TGFs in human amniotic fluid obtained during amniocentesis and in urine samples obtained from pregnant and normal donors was also pursued utilizing a modification of the above extraction procedure.

Major Findings:

1. Detection of transforming growth factors in mouse embryos. Acid/ethanol extracts, prepared from 12-13 day old embryos, have both EGF-competing activity and soft agar growth stimulating activity. All six mouse strains tested, (C3H/HEN, NIH (s), C57BL/6N, NIH(s)-+/Nu, AKR/N and Balb/c exhibited EGF competing activity ranging from a high value of 4.8 ng EGF equivalents/mg of protein for C57 (BL)/6N to a low value of 3.1 ng/mg for the AKR/N strain. Chromatography of Balb/c acid/ethanol extracts by gel filtration in acid conditions resolved two major peaks of EGF-competing activity with estimated molecular weights of 10K and 17K. In addition, a peak of EGF-competing activity eluted corresponding to normal mouse EGF. Both the 17K and 10K species support soft agar colony activity whereas no colonies were observed with the EGF-competing activity eluting in the region of mouse EGF (4-6K). The 17K EGF-competing activity forms very large, round colonies on the indicator cells, some with invasive protuberances while the smaller 10K activity supports the growth of small globular colonies. The 10K activity eluted during high pressure liquid column chromatography (HPLC) at a position different from mouse EGF.

2. Detection of transforming growth factors in the urine of pregnant human donors and in a patient with small cell carcinoma of the lung. A modification of the acid/ethanol extraction procedure was utilized to extract acid soluble polypeptides from urine samples. Both EGF-competing and soft agar growth promoting activity were detected after gel filtration of acid/ethanol extracted crude urine preparations. All urine samples tested contain human EGF (6K) and in addition an 8K variant which competes for EGF receptor sites but unlike human EGF supports soft agar colony growth. HPLC provides evidence that this 8K variant is different from EGF in its differential solvent elution profile. Two peaks of EGF-competing activity, both of which also support soft agar stimulating growth activity with estimated molecular weights similar to that seen in the fetus, are also seen in acid/ethanol extracts of urine from pregnant donors. Again the major human 6K EGF-competing activity, which elutes on HPLC in the region of mouse EGF prepared from submaxillary glands, does not support soft agar colony growth. Similar analysis of urine collected from a patient with small cell carcinoma of the lung also demonstrated transforming growth factor activity.

Significance to Biomedical Research and the Program of the Institute:

Many cellular gene products expressed during malignant transformation are also produced during differentiation and development. If, indeed, TGFs identical or similar to SGFs are expressed and functional during embryogenesis, this provides an excellent model system to both study and define those mechanisms involved in regulating the expression of the phenotypic responses observed during malignant transformation. Application of the methodology discussed to the detection of TGFs in the urine of cancer patients suggests the feasibility of examining the value of these factors as both diagnostic and prognostic indicators in the cancer patient.

Proposed Course:

Embryonic transforming growth factors (ETGF) will be purified from large scale embryo pools and structurally compared to the SGF isolated from MuSV-transformed cells. The mechanism(s) by which EGTF initiates the expression of the malignant phenotype will also be explored in conjunction with studies to evaluate the expression of TGFs in the urine.

Publications:

Twardzik, D.R., Reed, C.D., Weislow, O.S., and Fowler, A.K.: The effect of local anesthetics on cell surface receptors for the major envelope glycoprotein of murine leukemia virus. Int. J. Immunopharmacol. 2: 111-116, 1980.

Hellman, K.B., Brewer, P.P., Twardzik, D.R., and Hellman, A.: Protease Inhibitors modify tumor virus induction. Proc. Soc. Exp. Biol. Med. 166: 28-34, 1981.

Weislow, O.S., Fisher, O.U., Twardzik, D.R., Hellman, A., and Fowler, A.K.: Depression of mitogenic-induced lymphocyte blastogenesis by baboon endogenous retrovirus associated components. Proc. Soc. Exp. Biol. Med. 166: 522-527, 1981.

Twardzik, D.R., Ranchalis, J.E., and Fowler, A.K.: Interaction of murine thymocyte histocompatibility antigens with envelope glycoproteins of Rauscher murine leukemia virus. In Lapin, Boris (Ed.), Comparative Leukemia Research, Russia Publishing Firm, U.S.S.R., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05179-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Transfer of Gene(s) into Animal Cells Using Retroviruses as Eukaryotic Vectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Cha-Mer Wei	Expert	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	1.0	0.20

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have constructed and isolated a recombinant retrovirus containing the src gene of Harvey murine sarcoma virus (Ha-MuSV) and the thymidine kinase gene (TK) of Herpes simplex virus type 1 (HSV-1). The new viruses can induce focus formation on NIH 3T3 cells and convert NIH 3T3 (TK$^-$) cells into the TK$^+$ phenotype by carrying into the TK$^-$ cells the HSV-1-tk gene. In the TK$^+$ transformants, HSV-1 specific thymidine kinase can be identified by immunoassays. Hybridization analysis indicates that the recombinant virus contains both the Ha-MuSV src sequence and the tk gene sequence in a single RNA species of approximately 4.9 kilobases. We conclude that retroviruses can be used as true vectors for genes other than genes that lead to oncogenesis. We have also lined the HSV-1 tk gene to the deleted Moloney murine leukemia virus (Mo-MuLV) genome in double recombinants. The mouse TK$^-$ cells can be transformed into the TK$^+$ phenotype with much higher efficiency by transfection using recombinant DNA containing both the tk and the defective Mo-MuLV than by using the tk DNA alone.

PHS-6040
(Rev. 2-81)

Position Description

Objectives:

In order to gain insight into the genomic organization and gene expression of murine RNA tumor viruses in mammalian cells, one approach is to use recombinant DNA technology to obtain DNA copies of the RNA tumor viruses. The availability of such cloned DNA molecules enable one to study the viral genes at the nucleotide level and to manipulate the genomes at strategic regions which are involved in tumorigenesis and leukemogenesis. Experiments include construction of point and deletion mutants at these regions in vitro. Similar approaches could be employed to examine other important viral functions including sequence recognition for integration and gene expression at the transcriptional level. To study the expression of a particular gene in animal cells, one has to transfer the cloned gene back into a homologous or heterologous host cell. One of the approaches to this gene transfer is to introduce the naked DNA into an appropriate cell; another is to use an animal virus as the eukaryotic vector providing that this vector is not lytic or lethal to the host cell. Retroviruses will provide an additional animal virus vector system. The knowledge and techniques acquired from gene transfer research could be applied to correct genetic disorders in animals or humans.

Methods Employed:

Southern's gel blotting technique to identify viral DNAs in a mixture of cellular DNAs; RPC-5 column chromatography to fractionate DNA fragments according to their AT-GC contents and sizes; Analytical agarose gel electrophoresis to analyze DNA fragments; Preparative agarose gel electrophoresis to separate DNA fragments based on molecular size; Molecular cloning system using lambda phage as vector to obtain viral DNA sequences; Calcium-phosphate dependent transfection assay to assay for transforming and lytic functions of the DNA molecules; and Northern blotting technique to identify viral RNAs in viruses or animal cells.

Major Findings:

1. Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of Herpes simplex virus type I. We have constructed recombinants containing both the Harvey murine sarcoma virus (Ha-MuSV) genome and the thymidine kinase gene (tk) of Herpes simplex virus type 1 (HSV-1) linked to each other. The TK gene was placed in a position downstream from the long terminal repeat (LTR) and the src gene of Ha-MuSV. The DNAs from the λ recombinants were used to transfect NIH 3T3 mouse fibroblasts in order to obtain Ha-MuSV DNA-induced foci of transformed cells. The transformed foci were superinfected with a helper-independent retrovirus, and from the superinfected foci new individual retroviruses were isolated. The new viruses can induce focus formation on NIH 3T3 cells and convert NIH 3T3 (TK$^-$) cells into the TK$^+$ phenotype by carrying into the TK$^-$ cells the HSV-1 tk gene. From virus-infected cells, we have isolated nonproducer foci on NIH 3T3 and TK$^+$ transformants on NIH 3T3 (TK$^-$) cells containing one such new viral genome coding for the dual properties. The new retroviral sequence in the nonproducer cells can be rescued into virus particles at high titers by super

infection with a helper independent retrovirus. Hybridization analysis indicates that the recombinant virus contains both the Ha-MuSV src sequence and the tk gene sequence in a single RNA species of approvimately 4.9 kilobases. We conclude that retroviruses can be used as true vectors for genes other than genes that lead to oncogenesis.

2. Long terminal repeats of Moloney murine leukemia virus may be responsible for increased efficiency of transformation by the thymidine kinase gene of Herpes simplex virus type I. We also constructed double recombinants containing both the deleted Moloney murine leukemia virus (Mo-MuLV) genome and the thymidine kinase gene of HSV-1. The tk gene was placed in a position upstream from the LTR. We isolated one λ recombinant containing a Mo-MuLV promoter and a TK promoter in tandem position, another λ recombinant with two promoters opposed. During DNA transfection experiments, the DNAs isolated from either recombinant have higher efficiency for transforming NIH 3T3 (TK^-) into the TK^+ phenotype than transformation by the tk DNA alone. Since the tk gene will not be transcribed by the linked Mo-MuLV promoter without gene rearrangement in vivo, we suggest that the high efficiency of transformation may be due to more specific and more efficient integration of the transfected DNAs.

Significance to Biomedical Research and the Program of the Institute:

Application of the recombinant DNA technology to RNA tumor viruses would add a new dimension to our understanding of the organization of viral genomes and their biological functions. The methodology developed and biological functions revealed from studies with DNA viruses could be applied to RNA tumor viruses by analyzing their DNA copies from molecular cloning. It is now possible to construct deletion mutants in vitro at defined positions. When these mutants become available, it should be possible to analyze in detail the DNA sequences which are involved in integration, replication and further expression of viral genes. We have isolated a transmissible retrovirus containing both the src gene and the TK gene. The recombinant Ha-MuSV containing an extra genetic marker may be used to study the regulation of expression of either the transforming gene of Ha-MuSV or the TK gene. Since neither gene is essential for viral replication, one can potentially analyze for unusual mutants from one gene while monitoring their existence through the functional activity of the other gene.

Proposed Course:

To employ recombinant DNA technology to study the molecular biology of murine RNA tumor viruses. Among these viruses, Ha-MuSV, Mo-MuLV, Kirsten-MuSV and endogenous viral sequences are particularly interesting to us. The approach is to isolate proviruses including linear, circular and integrated forms of viral DNAs from infected cells. These DNA molecules, after being properly enriched and tailored, will be cloned in various lambda-E. coli systems. When these DNA sequences become available the following studies will be carried out: (1) Complete nucleotide sequences of these viruses. These experiments will be performed in collaboration with other laboratories using recently developed DNA sequencing techniques. (2) Construction of mutants. Viral mutants containing specific deletions and point mutations will be constructed from the cloned DNA molecules by modifying these molecules with restriction endonucleases and chemically synthesized linkers. (3) Viral gene expression. Many cloned viral DNA molecules are demonstrated to be biologically active using transfection assays. By examining the deletion location and specific mutations of the mutants constructed, it is possible to define sequences at a nucleotide level that are involved in integration, transcription, RNA processing, tumorigenicity and leukemogenicity. (4) Cellular sequences at the viral integration site. By cloning the neighboring genes or DNA sequences adjacent to viral integration sites, the possible regulatory signals and/or structural relationship between cellular and viral genes will be examined. (5) Continue to study the recombinant retrovirus containing both the *src* gene and the thymidine kinase gene. The regulation of expression of both the *src* and the thymidine kinase gene which are under the control of two different promoters will be investigated. The proviral genomes from infected cells will be cloned. (6) Continue to develop retroviruses as eukaryotic vectors to accomodate foreign genes in gene transfer research. Hopefully, the knowledge and techniques acquired from these studies could be applied to "gene therapy", i.e., to transfer the cloned normal gene(s) into patients to correct genetic disorder such as sickle cell anemia and thalassemia.

Publications:

Chang, E.H., Maryak, J.M., Wei, C., Shih, T.Y., Shober, R., Cheung, H.L., Ellis, R.W., Hager, G.L., Scolnick, E.M., and Lowy, D.R.: Functional organization of the Harvey murine sarcoma virus genome. J. Virol. 35: 76-92, 1980.

Wei, C., Lowy, D.R., and Scolnick, E.M.: Mapping of the transforming region of Harvey sarcoma virus genome using insertion-deletion mutants constructed *in vitro*. Proc. Natl. Acad. Sci. USA 77: 4674-4678, 1980.

Wei, C., Gibson, M.L., Spear, P., and Scolnick, E.M.: Construction and isolation of a transmissible retrovirus containing the *src* gene of Harvey murine sarcoma virus and the thymidine kinase gene of Herpes simplex virus type I. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05180-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Evolution and Sequence Organization of Mammalian Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Raoul E. Benveniste	Microbiologist	LVC	NCI
OTHER:	Nara Battula	Visiting Associate	LVC	NCI
	Stephen J. O'Brien	Geneticist	LVC	NCI
	George J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.15	1.25	0.90

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nucleic acid hybridization studies using cloned retroviruses, DNA's and recombinant DNA techniques are being used to examine the sites of integration and organization of viral sequences in cells and tissues. The principal goal of these studies is to develop probes that will allow the detection of RNA tumor viral sequences in normal and malignant human tissue. These same techniques are being employed to examine the organization of the two endogenous feline viruses (RD and FeLV) in various backcrossed animals which are offsprings of a genetic cross between an F_1 hybrid and a virogene-negative parent (leopard cat).

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To study the evolution and organization of endogenous primate and feline retroviruses within the mammalian genome. Recombinant DNA techniques will be employed to develop probes appropriate for the detection of virus-related sequences in human cells and tissues. In addition, the factors involved in the expression of primate viral sequences will be investigated.

Methods Employed:

The viruses being used are the Old World monkey isolates from this laboratory: the baboon type C virus, the colobus-macaque-rhesus class of type C virus, and the langur type D virus. The feline viruses include RD-114 and FeLV. Primary cell lines from various feline and ape species are being developed and maintained. These lines are used in virus isolation, host range, and viral interference experiments, as well as to study the control of viral transcription. Replication of retroviruses is detected by assaying the pellet obtained after high speed centrifugation of supernatant fluid from cells for reverse transcriptase activity. Radioimmunoassays for various type C viral proteins are also employed to characterize new isolates. The cloning of proviral DNA is performed using various plasmids as vectors as well as phages constructed from lambda. Restriction enzyme maps are generated for various retroviruses. The cloned proviral DNA can then be used as probes for cloning viral sequences integrated in the genomes of mammalian species. The expression of primate retroviral sequences in cells is being investigated with RNA blotting procedures after selection of poly A - containing molecules by oligo dT cellulose chromatography.

Major Findings:

1. Characterization of endogenous primate retrovirus sequences in monkey and ape tissues. The three classes of Old World monkey virus isolates include the baboon type C virus, the colobus-macaque-rhesus group of type C viruses, and the langur type D virus. It has previously been shown that endogenous viral DNA sequences related to these three classes of viruses are present in most primate cellular DNA. The divergence of single copy cellular DNA between the primate species can be used as the framework for the evaluation of the evolution of virus-related sequences. Sequences related to both type C families appear to diverge more rapidly in the Asian primates than in African primates. For example, related sequences could be readily detected by liquid hybridization techniques in the African apes (chimpanzee and gorilla) but only very low levels of hybridization were present in the Asian apes (orangutan or gibbon) or in human tissues. The type D sequences diverge so rapidly that we cannot detect related sequences in any of the apes or in man. The data suggested that viral nucleic acid sequences related to all three classes of Old World primate viruses are present in gibbon, orangutan and human tissues, but possess too little nucleic acid homology to be detected by these methods.

By using cloned, restricted fragments of the baboon endogenous type C virus and blot hybridization techniques we have detected related nucleic acid sequences in all ape as well as human tissues. The Asian apes (including man) seem to posses sequences (as defined by restriction enzyme analysis) that are not present in African ape tissue. We are currently cloning these human type C viral fragments in order to generate probes and to sequence the human virus.

There is one Old World monkey species, the colobus, in which it is possible to readily detect sequences related to all three classes of Old World retroviruses. Because of its phylogenetic position, the colobus is the only Old World genus where both integration and expression of these three virus classes can be studied; each class is distinct and present in 30-100 copies in the colobus genome. We are currently examing the control of transcription of RNA tumor virus genes in this species.

2. Detection of nucleic acid sequences related to primate viral genes in normal and malignant human cells. We are examining a variety of normal and malignant human tissues for the presence of primate retrovirus sequences using blot hybridization techniques with a probe prepared from a restricted fragment of the baboon virus. Since multiple fragments of the baboon and colobus type C viruses have been restricted and cloned in various vectors, we can examine whether certain portions of the genome are more conserved and preferentially expressed in human tumors.

In addition, we are attempting to isolate retroviruses from ape cells (gibbon, orangutan, chimpanzee and gorilla) since they are man's closest relatives. Human tissue has previously been cocultivated extensively in attempts to isolate retroviruses. Ape tissue has been much less extensively cocultivated, and since many retroviruses are rare one-time isolates (for example rhesus, colobus and langur viruses), an attempt at virus isolation from these species is justified.

3. Segregation of endogenous type C and feline leukemia viral genes in crosses between domestic cat and leopard cat. The domestic cat *Felis catus* contains two classes of RNA tumor viruses, feline leukemia virus (FeLV) and RD-114 type C viruses. The former class causes lymphoma and myeloproliferative disease while the latter class has not yet been shown to cause disease in cats. Both sets of viruses are found in multiple copies in the cellular DNA and are inherited as stable mendelian units from one generation to the next. The leopard cat from Southeast Asia, *Felis bengalensis*, does not contain either virus in its DNA. The offspring of matings between the leopard and domestic cat thus afford a unique opportunity to study the segregation of both sets of virogenes in F_1 hybrids and in the progeny of a backcross to the virogene-negative parent. Blot hybridization data with cloned RD-114 and FeLV virus are being analyzed to examine the frequency and distribution of both viral sequences in the various backcrossed cats. These results will be correlated with virologic data on the ability of various cell lines established from these animals to restrict the growth of RD-114 and FeLV.

Significance to Biomedical Research and the Program of the Institute:

The cocultivation techniques which have resulted in the isolation of various endogenous mammalian viruses should be applicable to the isolation of additional virus from man's closest relatives, the apes. It has been shown that endogenous retroviral DNA sequences are present in primate cellular DNA and are transmitted through the germ line. The viral sequences are therefore subject to the same evolutionary pressures as the cellular DNA sequences. The techniques described here will allow the detection of RNA tumor virus related sequences. The cloning of viral sequences is a low cost method that will provide large quantities of new viral DNA sequences whose biological activity can be studied at the molecular and cellular levels. Specifically the cloning of human type C viral genes will allow the study of the significance of this group of viruses in human neoplasia.

Proposed Course:

The data indicate that human tissues contain readily detectable type C viral gene sequences. In order to fully characterize this virus, we are attempting to clone these human viral sequences.

Hybrid leopard - domestic cats that contain a variable number of virogene copies will allow the study of the effects of gene dose on susceptibility and resistance to diseases mediated by this group of viruses. The knowledge gained from these tumor virus genome genetic transmission studies might also help us to better understand human neoplasia.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05202-01 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure, Function, and Utilization of Eukaryotic Promoter Sequences

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George E. Mark	Expert	LVC	NCI
OTHER:	Tom I. Bonner	Expert	LVC	NCI
	Edward H. Birkenmeier	Staff Fellow	LVC	NCI
	Robert Callahan	Biologist	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.05	0.95	0.10

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure, function and utilization of eukaryotic promoter sequences required for the specific initiation of RNA transcription are being studied, The endogenous retrovirus CPC-1 isolated from Colobus polykomos kidney cells provides a unique probe to dissect the modulators of eukaryotic gene expression. Specific goals are (1) to elucidate the specific DNA signal sequences (promoters) which control efficient transcription; (2) to ascertain the nature of the factors which modulate recognition of transcriptionally active genes; (3) to develope efficient eukaryotic cloning vectors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

Eukaryotic cells are capable of controlling the expression of at least a part of their genome by regulation of RNA transcription. Such specialized genes as those coding for ovalbumin, hemoglobin, and immumoglobulins have been shown to be under such control, as have the sequences for endogenous avian retroviruses. The mechanism(s) which account for the switching on and off of genes during development are poorly understood. Since prokaryotic gene regulation involves the efficiency with which RNA polymerase recognizes and interacts with specific DNA signal sequences (promoters), we may speculate that this interaction is also important in the eukaryote.

The endogenous (CPC-1) virus isolated from Colobus polykomos following long term cocultivation with a human carcinoma cell line A549 provides a probe to dissect the modulators of eukaryotic gene expression. Enigmatically, CPC-1 exists in colobus monkey tissue as a repressed gene, in spite of the fact that 50 to 70 copies are present. CrT analysis has shown that none of these genes are expressed as RNA transcripts. By defining the events which allowed the expression, and subsequent replication, of the virus genome we hope to gain a better understanding of those factors which switch on and off genes. Molecularly cloned CPC-1 and M432 DNAs (containing promoter sequences) have been used (1) to evaluate the sequences required for RNA polymerase II-directed transcription; (2) determined which factors modulate transcription; (3) and develop retrovirus-guided eukaryotic cloning vectors.

Methods Employed:

Functional promoter sequences have been cloned from both the endogenous murine virus M432 and colobus virus CPC-1 employing unintegrated DNA intermediates. The long terminal repeats were sequenced (Maxim and Gilbert method) to determine the primary sequence of presumptive promoter regions. Functionality of the CPC-1 promoter was examined by employing (1) the in vitro transcription system described by Manley et al, and (2) the expression enhancement of mouse sarcoma sequence in vivo. Subsequent subcloning in pBR322 of a 540 base pair HaeIII fragment containing the CPC-1 promoter was accomplished via EcoRI linkers. Truncated thymidine kinase specific DNA (both promoter and termination signals have been removed) will be inserted into the above CPC-1 subclone at the unique SacI site via SacI linkers and biological activity determined upon transfection of NIH 3T3 tk^- cells.

Major Findings:

1. The DNA sequences controlling transcription. The long terminal repeat derived from the replication competent CPC-1 was partially sequenced (Maxim and Gilbert) to determine the nature of its presumptive promoter region. The presence of two canonical "TATA" boxes was observed, 29 and 60 nucleotides upstream from the transcription initiation site (cap site; defined from the sequence of strong stop DNA). Also identified were two 9-nucleotide sequences (CCAATCATA) approximately 55 nucleotides upstream from each "TATA" box.

Whereas the later sequences may be involved in the binding of RNA polymerase and hence the efficiency of transcription, the former sequences most likely determine the cap site for transcription.

*** -121 -82 -60 -29 * +1 +56

·····CCAATCATA·····CCAATCATA·····TATAATAA·····TATATAA·····GAGCTC·····AATAA

Sac I

Potential Promoter I

53

Potential Promoter II

61

*location of base alterations
in endogenous sequences

The duplicitic nature of the promoter region suggested that its transcription efficiency might be quite high. This was substantiated by Crt analysis of productively infected A549 cells which revealed the presence of an extraordinarily high number of viral transcripts (5-10,000/cell) in these cells. Only the "TATA" box located at position -29 was used to initiate transcription in vivo which was determined from a S_1 analysis of poly $(A)^+$ total infected cell RNA. The activity and specificity of in vivo transcription was demonstrated in vitro employing restriction enzyme truncated cloned CPC-1 and a cell free extract. To ascertain the origin of the CPC-1 viral promoter sequences, two endogenous proviral LTRs were cloned from colobus kidney cells. Nucleotide sequence and in vitro transcription comparisons of the three DNAs revealed: (1) the CPC-1 promoter originated from colobus proviral sequences; (2) although both binding and initiation sites were unaltered both endogenous promoters were transcriptionally inactive. Computer-generated DNA secondary structures revealed striking similarities between the CPC-1 and M432 promoters (CCAAT and cap site both in denatured bubbles) whereas the inactive promoters formed different structures. We believe that the restriction of gene expression, depicted by endogenous CPC-1 sequences, may be mediated through nucleotide alterations of the relavent upstream promoter sequences.

2. The nature of transcription recognition factors. CPC-1 will replicate in only two established cell lines, neither of which is colobus kidney. Transfection of 3T3 cells by cloned CPC-1 DNA a mixture and cloned murine sarcoma DNA was unable to transform there cells. The reason for this result is unclear. Two possibilities present themselves. First, the consequence of RNA processing signals downstream from the CPC-1 promoter may be an unstable CPC-1/sarc transcript which is not translated. These signals have been removed by subcloning a 540 base pair Hae III fragment employing EcoRI linkers. Second, the CPC-1 promoter may require factors which are present in few cells. Cell-free transcription extracts, prepared from A549 cells (permissive) and colobus kidney cells (nonpermissive), and restriction enzyme truncated CPC-1 DNA are being used to define modulators of transcription.

3. Retrovirus LTRs hold a significant promise of being useful eukaryotic cloning vectors. To date the DNA sequence to be expressed has been placed either upstream or downstream from the LTR so as to use its transcription termination or initiation signals, respectively. The CPC-1 promoter has been found to be transcriptionally highly efficient. It also contains a unique SacI restriction site at the transcription cap site. Initially, MSV tk DNA which lacks promoter and terminator sequences is being ligated into the SacI site making use of SacI linkers. Eventually partially SacI-digested eukaryotic DNAs will be substituted within this LTR to take advantage of its promoter and termination (AATAA) signals.

Significance to Biomedical Research and the Program of the Institute:

The molecular mechanisms which underlie the regulation of transcription in eukaryotic cells are largely unknown. Understanding these mechanisms will lead to a broader comprehension of the factors which are able to modulate genes during cellular differentiation and neoplastic transformation. The importance of promoter insertion to enhance the expression of a restricted cellular gene has been established for avian leukosis and will undoubtably be extended to other oncogenic events. We are in a position to decipher some of the regulatory factors, while simultaneously applying the knowledge to obtain the expression of medically relevant genes.

Proposed Course:

CPC-1 promoter sequences provide a unique tool to define the mechanisms which regulate gene expression. These sequences will be used to: (1) more fully describe functional regions via determining the consequences of point initiations; (2) isolate cofactors which are required for the initiation of specific genes; and (3) to stably introduce new genetic information into eukaryotic cells.

Publications:

Lovinger, G.G., Mark, G.E., Todaro, G.J., and Schochetman: 5' Terminal nucleotide noncoding sequences of retroviruses: Relatedness of two old world primate type C viruses and airan spleen necrosis virus. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05211-01 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Etiology and Control of Human Breast Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul Levine	Medical Director	LVC	NCI
Other:	Jose Costa	Pathologist	LP	NCI
	Larry Muenz	Mathematical Statistician	BB	NCI
	Kamaraju Sreemahalakshmi	Visiting Fellow	LVC	NCI
	R. Gerald Suskind	Medical Director	LVC	NCI

COOPERATING UNITS (if any)

N. Mourali, Inst. Salah Azaiz, Tunis, Tunisia, and J.G. Bekesi, Mt. Sinai Hosp., New York, N.Y.

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Clinical Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Md. 21701

TOTAL MANYEARS: 0.65	PROFESSIONAL: 0.50	OTHER: 0.15

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is a multidisciplinary study of breast cancer in which epidemiologic, virologic, immunologic, and pathologic techniques are applied to the comparison of breast cancer in the United States and Tunisia.

Project Description

Objectives:

To investigate the pathogenesis and treatment of rapidly progressing breast carcinoma (RPBC) in Tunisia as a model for aggressive breast cancer in the United States.

Methods Employed:

More than 100 Tunisian breast tumors from patients seen at the Institute Salah Azaiz (ISA) were studied by pathologic, biochemical and immunologic techniques to determine differences between RPBC and non-RPBC cases. Immunologic tests evauated differences between Americans and Tunisians with and without breast cancer. Clinical and laboratory information on 112 patients entered into a chemotherapy study utilizing cyclophosphamide, methotrexate, and 5-fluorouracil were also analyzed. Hormone receptor assays were performed on 94 biopsies from Tunisian breast cancer patients and the results were compared with a series of biopsies from American women with breast cancer.

Major Findings:

1. Characterization of rapidly progressing breast cancer in Tunisia. Pathologic studies supported the concept of rapidly progressing breast cancer (RPBC) or pousee evolutive (PEV) as a separate biologic entity with the demonstration that even PEV-1, which has no clinical evidence of inflammation but which is based primarily on the patient's subjective report of rapid tumor growth, has a significantly higher frequency of nuclear grade 3 than the non-PEV (PEV-0) cases. The similarity between the more advanced RPBC cases and the so-called inflammatory breast carcinoma in the United States was demonstrated by the frequent finding of lymphatic permeation involving the skin. Hormone receptor studies demonstrated a correlation between progesterone receptor levels and a favorable response to treatment, but no differences between patients with and without RPBC were noted.

Significance to Biomedical Research and the Program of the Institute:

The development of a strong collaborative effort between the National Cancer Institute and the Institute Salah Azaiz in Tunisia is of great value in that it provides access to a group of patients with rapidly progressing breast cancer. Information gained as to the etiology and means of controlling this form of breast cancer in Tunisia can be expected to help in the control of fulminating breast cancer in the United States. The high frequency of RPBC in Tunisia allows information on this entity to be accumulated more rapidly than in the United States. Clinical and pathological data thus far indicate that RPBC in Tunisia is no different than fulminating breast cancer in the U.S., and therefore information obtained from the Tunisian patients would be directly applicable to breast cancer patients in the United States. The finding of the high content of antigen cross reacting with murine mammary tumor virus (MMTV) indicates that human material will be available that will accelerate studies on the involvement of viruses in the cause of breast cancer. The chemotherapy studies have already been of value to breast cancer patients in the United States and the results of these studies are encouraging American chemotherapists to treat patients with rapidly progressing breast cancer who in the past had not been treated with chemotherapy. The immunologic studies demonstrate the integrity of the immune system in patients with RPBC, providing guidelines to management of such patients.

Proposed Course:

A series of pathology slides will be obtained from various countries, particularly from Eygpt, to correlate the histopathologic features of breast cancer, the frequency of antigen cross-reacting with gp52 of MMTV and the clinical course of breast cancer patients. A comparative series including Eygpt, Tunisia and the United States will be of interest because of the general similarities between the Eygptian and Tunisian population, but the apparent absence of RPBC as a clinical entity in Egypt. Materials obtained for this study will also be available for further characterization of the antigen cross-reacting with gp52 and, in addition, should be a good source of material for new monoclonal antibodies being prepared to a variety of breast cancer related antigens. Studies of cellular and humoral immunity in breast cancer patients in Tunisia and the United States will continue with better characterized and more purified antigens. Epidemiologic data currently being obtained will be reviewed with an attempt to correlate changing patterns of living habits with an apparent change in the incidence of RPBC in Tunisia.

Publications:

Chan, S.H., Soares, N., Wallen, W., and Levine, P.H.: Detection of immunity to possible viral and tumor associated antigens in breast cancer and melanoma using the leukocyte inhibition assay. In Neiburgs, H.C. (Ed.): Third International Symposium of Prevention and Detection of Cancer. New York, Marcel Dekker, Inc., 1980, Vol. 2, Part II, pp. 1605-1612.

Mourali, N., Muenz, L.R., Tabbane, F., Belhassen, S., Bahi, J., and Levine, P.H.: Epidemiological features of rapidly progressing breast cancer in Tunisia. Cancer 46: 2741-2746, 1980.

Levine, P.H., Mourali, N., Tabbane, F., Loon, J., Terasaki, P., Tsang, P., and Bekesi, J.G.: Studies on the role of cellular immunity and genetics in the etiology of rapidly progressing breast cancer in Tunisia. Int. J. Cancer, in press.

Costa, J., Webber, B.L., Levine, P.H., Muenz, L., O'Conor, G.T., Tabbane, F., Belhassen, S., Kamaraju, L.S., and Mourali, N.: Histopathological features of rapidly progressing breast cancer in Tunisia. Int. J. Cancer, in press.

BEST DOCUMENT AVAILABLE

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05222-01 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunomicroscopic Localization of MTV and Other Viral Antigens in Mitotic Cells and Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Gerald Suskind	Medical Director	LVC	NCI
Other:	Ursula I. Heine	Research Microbiologist	LVC	NCI
	Paul H. Levine	Medical Director	LVC	NCI
	Jeffrey Schlom	Research Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

J. Hewetson, Litton Bionetics, Inc. (FCRC) Frederick, MD, and A. Demsey, Laboratory of Experimental Pathology, NIAMD, Bethesda, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.26	0.95	0.31

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods to determine the ultrastructural and immunomicroscopic localization of Murine Mammary Tumor virus antigens at intracellular sites using monoclonal antibodies as probes have been developed; immunoperoxidase and immunoferritin techniques under controlled conditions of fixation are used, and the relationship of these antigens to cytoskeletal proteins is under examination. Initial observations suggest an association of some antigens with the mitotic apparatus. Heterospecific determinants of MTV have been found in some mammary tumors and infected feline cell cultures, but not in murine cell lines derived from mammary adenomas.

BEST DOCUMENT AVAILABLE

Project Description

Objectives:

The association of microtubule organizing centers with certain retroviruses in mitotic cells has led us to investigate the possibility of a more common relationship of some retroviral proteins with elements of the cytoskeleton. (1) We aim: to localize, ultrastructurally, the sites of mammary tumor virus antigens using murine monoclonal antibodies to MTV as probes in cell cultures of homologous and heterologous species; to develop and apply immunomicroscopic techniques (immunoperoxidase) in conjunction with immunoelectron microscopy (immunoferritin) under controlled conditions of fixation that allow transmembranous penetration of antigen-antibody complexes; to determine whether these can be related morphologically and immunologically to the centriole and mitotic apparatus and cytoskeletal proteins; to investigate the presence of interspecific determinants of MTV antigens in cell cultures and in mammary tumors of homologous and heterologous species, including man, and to determine whether the presence of other viral antigens, e.g. the complement binding nuclear antigen of Epstein-Barr virus can be demonstrated in human cell cultures and human tumors (nasopharyngeal carcinoma, Burkitt's lymphoma) immunomicroscopically by using selective methods of fixation. The development of such a technique would be of considerable diagnostic and prognostic interest.

Methods Employed:

Immunomicroscopic procedures, such as indirect and enzyme coupled immunoperoxidase, immunofluorescence, light and transmission electron microscopy. Use of monoclonal antibodies to MTV of murine and Asian feral mouse species from murine hybridomas (obtained from Dr. Schlom).

Major Findings:

1. Cross reactivity with whole MTV antibody in mitotic spindle fibers. In the course of adapting immunoperoxidase procedures to tissue culture cell lines continuously infected with MTV, preliminary to developing immunoelectron microscopic techniques, we observed a persistent cross-reactivity with whole MTV antibody in mitotic spindle fibers of continuously infected feline cell cultures. This cross-reactivity was noted both in an indirect immunoperoxidase reaction and with an enzyme-coupled peroxidase-antiperoxidase procedure, using antibody reagents adsorbed with cytosol preparations of uninfected cells, but was absent in uninfected cells and in infected cells treated with non-immune serum. A gradation of peroxidase staining was also observed within phases of the mitotic cycle. These observations were found to be dependent on vigorous conditions of fixation and were noted only after brief fixation in dilute solutions of (para)formaldehyde or glutaraldehyde. Initial electron microscopic results, using an immunoferritin technique showed labeling of mature and budding virus particles, and possibly of some intracytoplasmic type A particles, using paraformaldehyde fixation. Under these conditions, ultrastructural preservation was, however, unsatisfactory.

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2. Immunomicroscopic and immunoelectron microscopic localization of monoclonal MTV antibodies. In continuing experiments we have tried both to develop methods of fixation adequate for electron microscopy that would allow antigenic preservation and intracytoplasmic penetration of immunoglobulin complexes, as well as to assay antibody probes of requisite specificity and reactivity by immunomicroscopy and immunoelectron microscopy. For this, we have employed a variety of monoclonal antibodies to MTV isolates from mus musculus and from several feral mouse species, produced by reactive clones of murine hybridomas, that were kindly provided by Dr. Schlom (Z01 CP 05136-01 LVC). In collaboration with Dr. Demsey (Z01 CP 05079-02 LVC), these have been initially characterized by immunoprecipitation and found to contain antigenic determinants coincident with gp52, gp36, as well as with p28 core protein. In trying a variety of bridging procedures with appropriate secondary antisera, both in murine and mammary tumors, in cell lines derived from murine mammary adenomas, or continuously infected feline cell lines, antigenic reactivity was found in three cell lines with only a few monoclonal antibodies. In murine mammary tumors heterospecific determinants to monoclonal IgG, antibodies to feral MTV isolates (Mus cookii) were found in a few tumor foci of mammary adenocarcinoma of C3H and RIII mice, which contain exogenous MTV, but not in tumors of BALB/c and C57BL1 mice. However, antigenic determinants were not found in established cell lines from homologous strains of mice. Also, monoclonal IgG, antibodies to MTV isolates from Mus cervicolor, were not reactive with a cell line (M-432) derived from that species, and in which precursor particles associated with the mitotic apparatus have been described. Some IgM antibodies to isolates from Mus musculus, however, were found to be reactive with two established cell lines derived from C3H mammary tumors. These contain abundant virus and precursor particles. Using fixation procedures developed by Willingham, et al., it was shown that the antigenic determinants, as well as ultrastructure of murine Mm T-73, Mm 5-mT cells or feline CrFK cells can be preserved by preventing the cross linking and polymerization of glutaraldehyde with a carbodiimide and enhancing membrane permeability with mild detergents.

Significance to Biomedical Research and the Program of the Institute:

Immunomicroscopic techniques, both at the level of the light- and electron microscopes suitable for studying the intracellular localization of viral antigens and their interaction with cytoskeletal proteins have been developed and adapted. Initial observations suggest a possible relationship of endogenous tumor virus antigens to the mitotic apparatus. These studies should be of relevance to an understanding of the cellular biology of transformation. A characterization of heterospecific determinants of MTV antigens may be of pathogenic or etiologic significance in the development of human mammary carcinoma.

Proposed Course:

It is proposed to explore further by immunoelectron microscopoy the cytoplasmic localization of MTV antigenic determinants of both core and envelope proteins, using mouse and rat monoclonal antibodies, and to study possible modification of these sites in mitotically arrested cells.

Publications:

U.I. Heine, R.G. Suskind, I. Margulies, and A.E. Demsey: Oncornavirus precursor particles and the microtubule organizing centers. Archiv. fuer Geshwulstforschung 50: 715-723, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05223-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of Mitogenesis in Tumor Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Nancy H. Colburn	Expert	LVC	NCI
OTHERS:	Marian Copley	Staff Fellow	LCP	NCI

COOPERATING UNITS (if any)

R. Tucker, John Hopkins Medical School, Baltimore, MD; H. Herschman, University of California, Los Angeles, CA

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.36	0.30	1.06

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

One of the major hypotheses for tumor promotion proposes that promoters function principally to stimulate proliferation of initiated cell populations which produces either 1) clonal selection of latent tumor cells or 2) increased probability of chromosomal events such as gene rearrangements or fixation of DNA damage. Recent work in our laboratory suggests that the promoting activity of phorol esters in JB6 cells is not due to a promoter-increased rate of cell division. This conclusion derives from two lines of evidence. The first is that when JB6 cells are exposed to promoter under conditions in which they cannot undergo promoter-dependent mitogenesis, promotion of tumor cell phenotype is not inhibited. The second is that variants of promotable JB6 cells which have been selected for resistance to the mitogenic activity of the phorbol ester TPA have been found to retain promotability, thus ruling out mitogenic stimulation as a required event in promotion of transformaion in JB6 cells. These TPA mitogen-resistant variants are currently being used to discover biochemical events which determine the mitogenic response.

Project Description

Objectives:

To determine whether promoter dependent mitogenic stimulation is required for promoting activity in JB6 cells. To determine whether promotion of anchorage independence in JB6 cells can occur under conditions in which promoter-induced mitogenesis does not and to determine whether cell lines selected from promotable JB6 lines for TPA mitogen resistance become nonpromotable.

Methods Employed:

Selection for mitogen resistance as described by Pruss and Hershcman. Assay of anchorage independence response and mitogenesis response by increased cell number and labeling index.

Major Findings:

1. Promotion of transformation without promoter-induced mitogenesis. Promotion of anchorage independence in JB6 cells occurs when cells are exposed to TPA only during logarithmic growth. Since TPA dependent mitogenesis only occurs in JB6 cells (and a variety of other cells) at plateau density as a release from quiescence, that this plateau density mitogenesis is not a requirement for promotion of anchorage independence in JB6 cells (Colburn and Ozanne, submitted for publicaiton).

2. Selection of mitogen-resistant JB6 cell lines. A promotable clone of JB6 cells has been subjected to a selection for resistance to TPA mitogenesis by exposure to TPA plus colchicine at plateau density (Pruss and Herschman). Under these conditions the sensitive cells are trapped in mitosis and washed off and the resistant cells remaining are then collected. After cloning, a number of cell lines which are completely resistant to TPA mitogenesis have been obtained including some which are transformed (anchorage independent) and some which are not (anchorage dependent).

3. TPA mitogen-resistant variants are promotable. We have now isolated three TPA mitogen resistant cell lines which are promotable to anchorage independence by TPA. This clearly rules out the TPA dependent release from quiescence type of mitogenesis which occurs at plateau density and presumably in various other growth inhibited states, as a requirement for promotion of transformation in JB6 cells.

Significance to Biomedical Research and the Program of the Institute:

Whether tumor promoters act primarily or entirely as mitogens to bring about tumor promotion has been for some time one of the major unanswered questions in carcinogenesis. Recent reports (Kennedy and Little; Peraino, et al.) have suggested that promotion can occur without mitogenesis. Now, this conclusion has been strengthened considerably by using an independent approach to demonstrate that TPA mitogen resistant clones are promotable.

Proposed Course:

To make use of cells of the various phenotypes derived from the selection experiment for mechanism studies. These include mitogen resistant promotable, non-promotable and transformants. The basis for resistance will be sought. To maintain stable clonal lines of each phenotype.

Publications:

Colburn, N.H., Wendel, E., and Abruzzo, G.: Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phrobol esters: Mitogen resistant variants are sensitive to promotion. Proc. Natl. Acad. Sci., in press.

Colburn, N.H., Dion, L.D., and Wendel, E.J.: The Role of Mitogenic Stimulation and Specific Glycoprotein Changes in the Mechanism of Late-stage Promotion in JB6 Epidermal Cell Lines. In Hecker, E., (Ed.): "Cocarcinogenesis and Biological Effects of Tumor Promoters. Raven Press, in press.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05224-01 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of Cellular Glycoprotein Shifts in Promotion of Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Louis D.Dion	Staff Fellow	LVC	NCI
OTHERS:	Nancy H. Colburn	Expert	LVC	NCI
	Beverly Peterkofsky	Research Chemist	LB	NCI
	Luigi De Luca	Supervisory Research Chemist	LEP	NCI
	Anton Jetten	Expert	LEP	NCI

COOPERATING UNITS (if any)

M. Sobel, Laboratory of Developmental Biology and Anamolies, National Institute of Dental Research, NIH, Bethesda, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.05	PROFESSIONAL: 0.90	OTHER: 0.15

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Since tumor promoters are nonmutagenic and membrane active, one hypothesis for the mechanism of tumor promotion proposes that the biological response is mediated by specific promoter-induced changes in cell surface glycoconjugate targets. Recent investigations in our laboratory have led to the discovery of three promoter-sensitive glycoproteins in JB6 cells having molecular weights 180,000, 150,000 and 220,000. The gp180 and gp150 have been identified as the pro-alpha-1 and pro-alpha-2 subunits of procollagen, respectively. Both phorbol and non-phorbol promoters such as epidermal growth factor and mezerein are active in producing these 3 glycoprotein decreases. TPA exposure switches off procollagen synthesis pretranslationlly (probably transcriptionally) as indicated by a lack of translatable collagen mRNA assayed in an in vitro protein synthesis system. Antipromoting concentrations of retinoic acid prevent the decreases in procollagen levels produced by promoter, but the level of retinoid action is postranslational. We propose that the transcriptional switch affecting collagen synthesis is indicative of a required event in promotion which is coordinately regulated with procollagen.

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Project Description

Objectives:

To demonstrate that tumor promoting phorbol esters selectively inhibit the synthesis of a small number of glycoproteins. Antipromoting retinoids antagonize this effect. The predominant effect among these phorbol ester sensitive glycoproteins is the rapid inhibition of mRNA synthesis for procollagen. The specificity and rapidity of this response suggests that it may be a direct effect of the TPA. Specifically to determine if (a) altered levels of procollagen synthesis are directly involved in the induction and/or maintainence of the transformed phenotype; (b) other major secreted macromolecules which are coordinately regulated with procollagen are also inhibited by TPA (the major secreted molecules of interest include fibronectin, laminin, and glycosaminoglycans); (c) the collagen genes share promoter sequences with other coordinately regulated genes which may be directly involved in the induction and/or maintainence of transformation (the collagen gene would in this case offer a convenient but indirect probe for the "promotion genes"); (d) the retinoid antagonism of TPA-induced promotion and the retinoid antagonism of TPA inhibition of procollagen are mechanistically related; and (e) decreased or altered collagen synthesis is related to sensitivity to TPA-induced promotion.

Methods Employed:

Collagen, laminin and fibronectin synthesis are determined by precursor incorporation and separation on SDS-PAGE, and when appropriate, immunoprecipitation. Collagen is quantitated by release of ^{14}C-proline after exhaustive digestion by purified collagenase.

Major Findings:

1. *TPA inhibition of procollagen synthesis*. The predominant effect of TPA on glycoprotein synthesis is the inhibition of a 180,000 molecular weight glycoprotein which has been identified as pro-alpha-1 procollagen. Also inhibited are the 150,000 pro-alpha-2 protein and a 220,000 mw glycoprotein which is partly composed of fibronectin. The inhibition of procollagen by TPA shows the correct analogue structure/activity response as determined by correlation with tumor promoting activity in vivo. Mezerein and epidermal growth factor are also active in inhibition of procollagen synthesis in JB6 cells. This demonstrates that the procollagen response is not limited to phorbol promoters alone. TPA acts rapidly to reduce the collagen mRNA synthesis and partial inhibition can be observed as quickly as 30 minutes. In vitro translation of isolated messenger RNA confirms that by 24 hours essentially no new collagen is being synthesized.

2. *The antipromoting retinoid retinoic acid markedly enhances the amount of cell-associated procollagen*. When given in conjunction with TPA there is an antagonism of the TPA-dependent reduction. The time course of the retinoic acid effect is slower than that of the TPA. The retinoid effect is observable by 24 hours, but is further enhanced by 72 hours. Retinoic acid does *not* enhance the transcription or translation rate of procollagen mRNA

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but does appear to increase the size of the cell pool of mature presecretory procollagen.

3. Loss of procollagen in promotable vs. nonpromotable cell lines. We have assayed promotable and nonpromotable clones of JB6 cells and have found that the promotable clones have a more pronounced loss of procollagen than do the nonpromotable clones. Of particular interest is JB6-C125 which is nonpromotable and displays a very fast recovery of procollagen such that procollagen synthesis at 48 hours has returned to close the control valve. Preliminary evidence also indicates that the nonpromotable clones produce a more highly mannosylated form of procollagen.

Significance to Biomedical Research and the Program of the Institute:

The question concerning the biochemical mechanism by which a nonmutagenic promoter irreversibly induce the transformed phenotype in preneoplastic cells is one of the important unanswered questions in carcinogenesis. Evidence for regulation at the level of transcription or secretion of macromolecules would provide new insights and approaches to this subject.

Proposed Course:

To characterize the major secreted macromolecules of JB6 and the TPA and RA effect on their secretion; to determine if colonies in agar have produced around themselves an extracellular matrix in part composed of collagen fibers, and if so are there distinctive differences between the promotable and nonpromotable clones; to investigate the mechanism whereby promoters inhibit procollagen transcription; and to investigate the target(s) of the antipromoting action of retinoids.

Publications:

Colburn, N.H., Dion, L.D., and Wendel, E.J.: The Role of Mitogenic Stimulation and Specific Glycoprotein Changes in the Mechanism of Late-stage Promotion in JB6 Epidermal Cell Lines. In Hecker, E. (Ed.): Cocarcinogenesis and Biological Effects of Tumor Promoters. Raven Press, in press.

Dion, L.D., DeLuca, L., and Colburn, N.H.: Phorbol ester-induced anchorage independence and its antagonism by retinoic acid correlates with altered expression of specific glycoproteins. Carcinogenesis, in press.

BEST DOCUMENT AVAILABLE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05225-01 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Gangliosides and Other Membrane Lipids: Possible Regulatory Role During Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Leela Srinivas	Visiting Fellow	LVC	NCI
OTHERS:	Thomas D. Gindhart	Expert	LCP	NCI
	Nancy H. Colburn	Expert	LVC	NCI
	Wayne Anderson	Research Chemist	LP	NCI

COOPERATING UNITS (if any)

P. Fishman, R. Brady, Developmental and Metabolic Neurology Branch, NINCDS, NIH, Bethesda, MD; G. Muschik, Litton Bionetics, Inc., (FCRC), Frederick, MD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.15	0.90	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Further testing of the hypothesis concerning the role of cellular glycoconjugates in promotion is focused on changes in cell surface glycolipids particularly gangliosides. The rationale for investigating these sialic acid containing glycolipids is that 1) phorbol esters are known to produce specific ganglioside changes under conditions in which they regulate expression of differentiation and 2) binding to specific surface gangliosides is required for the biological function of a number of regulators of cellular growth and differentiation including certain hormones. Recent results on this project have shown that tumor promoting but not nonpromoting phorbol esters produce specific changes in ganglioside synthesis principally a 10-fold decrease in the net synthesis of a trisialoganglioside (GT). This change is completely blocked by antipromoting concentrations of retinoic acid. Furthermore, JB6 variants which are promotable to tumor cell phenotype by phorbol esters consistently show the decrease while their nonpromotable counterparts do not, suggesting that a ganglioside shift may play a causal role in promotion of neoplastic transformation.

Project Description

Objectives:

The objective of this project is to demonstrate whether specific changes in cell membrane gangliosides and other lipids are involved in the process of promotion of transformation in JB6 cells. Specifically, to study the ganglioside profile of promotable JB6 mouse epidermal cells on exposure to phorbol esters and other tumor promoters; to study the correlation between activity for producing these changes and tumor promoting activity; to determine whether a target for the antipromoting action of retinoids is at the level of ganglioside synthesis; to study the mechanisms whereby phorbol esters and retinoids regulate ganglioside biosynthesis; to ascertain whether specific ganglioside changes may play a causal role in promotion of transformation by studying (a) the correlation of ganglioside response with promotability of variant cell lines, and (b) the possible promotion inhibitory effects of certain gangliosides on phorbol ester promotion.

Methods Employed:

Classical lipid extraction procedures, a thin layer chromatography system modified in our lab to give better resolution. A better ganglioside band visualization procedure was also developed.

Major Findings:

1. Tumor promoting phorbol esters produce specific changes in ganglioside biosynthesis. The de novo biosynthesis of gangliosides was studied by labelling the cells with D-1-^{14}C glucosamine for a terminal 4 hour period during a 24 hour TPA treatment. It was observed that TPA produces an 8- to 10-fold decrease in the biosynthesis of a trisialoganglioside (G_T). This was accompanied by a two-fold increase in an unknown ganglioside and a two-fold increase in GD1b. These three changes are observed from 24-48 hours post-TPA treatment and are brought about by a concentration of TPA reported by us to bring about promotion of anchorage independence in JB6 cells. The different phorbol esters ranked in their order of promoting activity elicit these specific changes in the gangliosides according to their potency.

2. Promoter-induced changes in ganglioside biosynthesis are antagonized by antipromoters and fail to occur in nonpromotable variants. The antipromoter retinoic acid completely antagonizes the changes brought about by TPA. Moreover, clonal variants of JB6 which are promotion-sensitive show a consistent drop in GT and increase in GD1b. Both responses are missing in the nonpromotable clones. The change in the unknown GX does not correlate with promotion sensitivity.

It has also been observed that when GT is incorporated into JB6 cells they can resist the morphological changes and the induction of anchorage independence brought about by TPA.

3. Role of neuraminidase in regulating GT switch. We have preliminary evidence to show that JB6 cells contain a neuraminidase with a substrate

preference for GT. Neuraminidase, a readily inducible enzyme, could be regulating the ganglioside concentrations in JB6 cells.

Significance to Biomedical Research and the Program of the Institute:

Since gangliosides have been shown to be important in cellular responses to a number of hormones, toxins and other environmental agents it appears reasonable to investigate their role in mediating responses to environmental carcinogens and promoters. It will be useful to learn whether ganglioside changes could be used as a marker for preneoplastic progression and if so whether specific reconstitutions would prevent the progression.

Proposed Course:

To study the regulation of ganglioside synthesis by promoters, we intend to study induction of neuraminidase by TPA. We already have evidence to show that potent neuraminidases exist in JB6 cells. Neuraminase characterization will be carried out; to determine whether the inhibition of tpa-induced anchorage independence is brought about specifically by gt or also by other gangliosides or other lipids; to determine whether other non-phorbol tumor promoters would also bring about these ganglioside changes; to determine whether these changes are still present in tumorigenic cells.

Publications:

Srinivas, L., and Colburn, N.H.: Tumor promoter induced ganglioside changes in promotable mouse spidermal cells: Antagonism by an antipromoter. J. Natl. Cancer Inst., in press.

Colburn, N.H., Srinivas, L., and Wendel, E.J.: Responses of preneoplastic epidermal cells to tumor promoters and growth factors: Use of promoter resistant variants for mechanism studies. J. Supramol. Struct. Cell. Biochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05226-01 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of Cell Surface Receptors in Regulating Biological Responses to Tumor Promoters

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Nancy H. Colburn	Expert	LVC	NCI
OTHERS:	Thomas D. Gindhart	Expert	LCP	NCI
	Bakul Dalal	Visiting Fellow	LCP	NCI
	George J. Todaro	Medical Officer	LVC	NCI
	Michael Sporn	Medical Officer	LC	NCI
	Anita Roberts	Staff Scientist	LC	NCI

COOPERATING UNITS (if any)

P.M. Blumberg, Department of Pharmacology, Harvard Medical School, Boston, MA; B. Magun, Department of Anatomy, University of Arizona, Tucson, AZ

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.15	PROFESSIONAL: 0.20	OTHER: 0.95

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Phorbol diester binding sites appear very stable biologically showing no significant variation in number, affinity for 3H-PDBu or down modulation among preneoplastic cells deliberately selected for resistance to TPA induced mitogenesis or promotion. Nor does malignant transformation detectably perturb these binding sites in mouse or human cells. Loss of EGF receptors correlates with loss of the mitogenic response to TPA in monolayer culture but has no effects on 3H-PDBu binding parameters or promotion of transformation. Transforming growth factor (TGF) from a human tumor line can promote anchorage independence in a TPA promotable mouse epidermal cell line lacking EGF receptors and responsiveness to EGF. Current studies seek to identify cell surface receptors for TGF distinct from EGF receptors on this cell line as well as inducibility of TGF secretion by tumor promoters.

Project Description

Objectives:

To determine the role of phorbol diester receptor number, binding affinity and down modulation in promotability of mouse epidermal cell lines by TPA; the relation between phorbol diester receptors and receptors for growth factors; the relation between phorbol diester receptors and transformation; the role of EGF receptors in TPA-induced mitogenesis and promotion; and the role of TGF in TPA-induced mitogenesis and promotion.

Methods Employed:

Classical Scatchard analysis of ^{3}H-PDBu binding to intact epidermal cells in monolayer culture. Induction of down modulation of ^{3}H-PDBu binding by preincubation with unlabeled PDBu.

Major Findings:

1. Phorbol diester binding sites are biologically stable. Mouse epidermal cell lines differing from each other in responsiveness to TPA with respect to mitogenicity in monolayer culture, promotability in soft agar and even transformation were all found to possess approximately the same number of phorbol diester binding sites of similar affinity and parallel patterns of down modulation of ^{3}H-PDBu binding. Variant lines lacking receptors for epidermal growth factor (EGF) did not differ from cell lines possessing EGF receptors with respect to any of the above parameters of phorbol diester binding.

2. Cell density regulates phorbol diester binding sites. A physiologic means of regulating phorbol diester number has been found which offers a novel approach to identifying the endogenous ligand for this binding site. All of the JB6 epidermal cell derivatives show about a fourfold decrease in number of binding sites when they pass from log phase growth to plateau phase. In contrast the number of EGFR increase significantly at plateau density indicating divergant mechanisms regulating these two different receptors.

3. EGF may mediate the mitogenic but not the promotion response to TPA. Variant lines lacking EGF receptors do not give a mitogenic response to TPA but can undergo promotion in response to TPA indicating that EGF may mediate the mitogenic effect of TPA on JB6 derivatives but not the promotion response. One line lacking EGF receptors which undergoes promotion in response to TPA is promotable by TGF but not EGF suggesting that TGF and other growth factors may act through receptors different from the EGF receptor.

Significance to Biomedical Research and the Program of the Institute:

The molecules which represent the specific phorbol diester binding sites appear to be very stable in response to deliberate efforts to select for variant cells unresponsive to the mitogenic or promoting effects of TPA. Despite the multitude of biochemical systems which do change with transformation the phorbol diester binding site remains unaffected which suggests that it is part of a basic physiologic mechanism whose functioning is essential for cell survival. Attempts to control preneoplastic progression or tumor growth through pharmacologic blockade of this binding site would be expected to encounter severe, undesirable side effects since every mammalian cell type tested to date except erythrocytes possesses these binding sites.

Proposed Course:

Continuation of these studies towards the objectives as stated above.

Publications:

Colburn, N., Gindhart, T., Dalal, B., Hegamyer, G., Blumberg, P., and Delclos, B.: The Role of Phorbol Ester Binding and Down Modulation in Responses to Promoters by Mouse and Human Cells. In Rice, J. (Ed.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum, in press.

Colburn, N.H., Srinivas, L., and Wendel, E.J.: Responses of preneoplastic epidermal cells to promoters and growth factors: Use of promoter resistant variants for mechanism studies. J. Supramol. Struct. Cell. Biochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05227-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Signal Transduction Triggered by Promoter-Receptor Binding

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Nancy H. Colburn	Expert	LVC	NCI
OTHERS:	Thomas D. Gindhart	Expert	LCP	NCI
	Bakul Dalal	Visiting Fellow	LCP	NCI
	Louis D. Dion	Staff Fellow	LVC	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 0.45	PROFESSIONAL: 0.30	OTHER: 0.15

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to identify the events triggered by binding of phorbol diesters to their specific binding sites which actually lead to tumor promotion rather than epiphenomena. The roles of membrane lipid changes, reactive oxygen and monovalent cation transport have been probed and each has been found to be promotion relevant. Inhibitors of the phospholipid methyltransferase system, 3 DZA and SAH can block TPA-induced promotion in JB6 cells. H2O2 alone is a known promoter in vivo and in our in vitro system. Catalase preparations have abrogated TPA-induced changes in morphology, procollagen secretion and promotion itself. Arginine vasopressin, which enhances the mitogenic effect of several growth factors by inducing sodium influx, has induced anchorage independent growth. In each case, studies are in progress to definitively relate the expected mediators to promotion and to each other.

Project Description

Objectives:

The objective of this research is to characterize the biochemical effector mechanism of the phorbol diester binding site. Identification of the events which occur most immediately following occupancy of the binding site is the initial objective and clear separation of promotion - relevant distal events from those unrelated to tumor promotion are the final objectives. Specific studies are aimed to determine sensitivity of phorbol diester induced promotion to inhibitors of (a) phospholipid methyltransferases (3-deazadenosine and S-adenosylhomocysteine); (b) reactive oxygen (catalase): H2O2 has previously been shown to be a promotor in this system in our laboratory; and (c) sodium ion transport (amiloride). The search for specific gene products induced by TPA in the human myeloid leukemia line K562 which responds to TPA by undergoing myelomonocytic differentiation is also being pursued.

Methods Employed:

Addition of putative agonists or antagonists of TPA induced tumor promotion to our standard soft agar assay.

Major Findings:

1. 3-deazadenosine (3DZA) inhibited TPA induced promotion. S-adenosylhomocysteine, which potentiates the ability of 3DZA to inhibit phospholipid methyltransferases in the RBL-1 histamine release system did not potentiate 3DZA inhibition of tumor promotion.

2. Catalase blocking of TPA promotion. Catalase completely blocked TPA-induced promotion as well as the morphologic change usually induced by TPA in epidermal cells in monolayer culture.

3. TPA induction of a 55K cellular protein in human leukemic cells. TPA induces synthesis of a 55K major cellular protein in the human leukemic cell line K562. This event occurs at picomolar concentrations of TPA, correlates with induction of resistance to natural killer lymphocyte activity and may be mediated by an unusually high affinity receptor for TPA found in human myeloid cells.

4. Vasopressin induction of anchorage independence. Arginine vasopressin, which induces a sodium ion influx and synergistically enhances responses to growth factors in many cells has induced anchorage independent growth in initial experiments.

Significance to Biomedical Research and the Program of the Institute:

Phospholipid methylation, reactive oxygen generation and sodium ion influxes may all be involved in tumor promotion. Manipulation of these systems appear to offer more promising new approaches to cancer control than blockade of the phorbol diester binding site.

Proposed Course:

Characterization of changes in cell membrane phospholipid composition induced by tumor promotors and search for correlation of observed changes with promotion. Specific lipids modulate the functioning of many membrane bound enzymes and receptors. Confirmation that all of the promotion blocking ability of catalase preparations used to date is due only to enzymatic degradation of H_2O_2 produced by epidermal cells in response to TPA. Identify the target molecules attacked by H_2O_2. Determination of whether arginine vasopressin promotion is attributable only to induction of sodium influx by attempting inhibition with the sodium ion channel blocking diuretic amiloride and induction of sodium influx by the antibiotic monesin which acts as a sodium prefering monovalent ionophore independent of the normal sodium ion channel. Characterization of changes in protein phosphorylations induced by tumor promotors in promotable and nonpromotable cell lines.

Publications:

None. Project recently initiated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05228-01 LVC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genes for Promotability

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Nancy H. Colburn	Expert	LVC	NCI
OTHERS:	Thomas D. Gindhart	Expert	LCP	NCI
	Ulf Rapp	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

H. Young, Litton Bionetics, Inc., (FCRC), Frederick, MD; J. Greene, Johns Hopkins University, Baltimore, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.15	0.10	0.05

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The overall objective of this research is to identify the changes in the genetic material itself or its regulation which must occur for mammalian cells to undergo preneoplastic progression in response to tumor promoters. Recent results from our laboratory indicate that promotability behaves as a dominant trait. Complementation analysis is being carried out with the aim of estimating the number of different genes (and gene products) involved in determining promotability. In addition, cloning of the relevant genetic material and analysis of its properties in direct DNA transfection experiments can now be performed.

Project Description

Objectives:

To determine the number and nature of genetically distinct steps required for tumor promoting phorbol diesters to induce anchorage independent growth in the JB6 family of epidermal cell lines. Characterization of the genetic loci in terms of their function(s), regulation and structure will be conducted. Gene cloning, sequencing and identification of regulatory mechanisms at the DNA level.

Methods Employed:

Polyethylene glycol induced fusion techniques are being used to create somatic cell hybrids between promotable and nonpromotable variant lines as well as between different nonpromotable lines. Desired fusion products will be separated from unfused cells and undesired fusion products using fluorescent beads and a Becton-Dickinson FACS IV cell sorter. Transfection experiments will employ current methods of generating restriction fragments of DNA, amplification in bacteria and $CaPO_4$ precipitation.

Major Findings:

1. Promotability behaves as a dominant genetic characteristic. Promotability behaves as a dominant trait in fusion experiments to date using promotable and nonpromotable parent cell lines. Polyethylene glycol treatment does not affect the soft agar assay.

2. Complementation Analysis of Promotion. Nonpromotable variant cell lines have failed to complement each other indicating that a single genetic locus or set of linked loci determines promotability in the clone 41 series of TPA promotion resistant variants.

3. Effects of viral DNA on the promotion response to TPA. Cellular DNA sequences associated with viral DNA isolated from transformed cells has not conferred promotability on otherwise nonpromotable cells. Collaboration with Ulf Rapp, LVC, NCI.

Infection of cells with viruses from promotable rat cells has not rendered recipient cells susceptible to TPA elicited promotion. Collaboration with Howard Young, Litton Bionetics, Inc., FCRC.

4. Fusion of tumorigenic with non-tumorigenic cells. Tumor cells show enhanced anchorage independent growth when fused with normal cells. This effect is not seen when tumor cells are simply mixed with normal cells. That the transformed phenotype behaves as a dominant trait corroborates the dominance of promotability.

Significance to Biomedical Research and the Program of the Institute:

Identification of genetic information required for inducibility of preneoplastic progression should lead to improved mechanisms of tumor prevention and tumor control.

Proposed Course:

Direct DNA transfection experiments can now be conducted since promotability behaves as a dominant trait in this system. Pursuit of the final objectives as stated above is the proposed course of this project.

Publications:

None. Project recently initiated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05229-01 LVC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Production of Monoclonal Antibodies to Viral Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Fulvia Veronese	Visiting Fellow	LVC	NCI
OTHER:	John R. Stephenson	Visiting Scientist	LVC	NCI
	Gary J. Kelloff	Senior Surgeon	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The transforming proteins encoded by the type C virus, but clearly of host cell origin, provide potential transplantation antigens that will understandably be of direct importance in cancer. This project is focused on the production of monoclonal antibodies to these proteins that will result in the evaluation of these proteins as transplantable antigens; a determination of their presence throughout the phylogenetic scale; and a better understanding of the mechanisms of transformation and thus the means to biochemically or immunologically alter the events that lead to cell transformation.

Project Description

Objectives:

Production of hybridomas made from immune lymphocytes obtained from rats immune to syngeneic tumor cell lines containing the polyprotein of the feline sarcoma virus(es) or the Abelson leukemia virus. The hybridomas are to provide a library of high-titered monospecific antibodies to the different antigenic determinants of the transforming proteins.

Methods Employed:

Cell culture techniques include maintenance of myeloma lines, cell fusions of myeloma cells with immune lymphocytes, cell cloning, and production of monoclonal hybridomas. Immunological procedures include immunoprecipitation and gel electrophoresis.

Major Findings:

Syngeneic cell lines have been derived from embryonic cells of the inbred Fisher rat so that the host's immune response could be examined against such cell lines nonproductively transformed by certain type C virus(es). These viruses encode as one of their major translational products, polyproteins with transforming activity; such proteins presumably of host cell origin. Cell lines under study include nonproducer cells transformed by FeSV, Abelson leukemia virus (AbLV) and BALB/c sarcoma virus (B/c-MSV). These various nonproducer clones differ in the types and levels of the gag structural proteins that are produced, but most lines under study have the transforming proteins. Animals hyperimmunized with nonproducer transformed cells are able to regress the syngeneic tumor challenge and antisera obtained from such animals have been shown by polyacrylamide gel electrophoresis to be reactive with the structural and nonstructural portions of the polyproteins. These antiserum reveal that the polyprotein is immunologically active at the cell surface in viable cells as determined by a chromium release cytotoxicity assay. These regressor rats have also been shown to have immune lymphocytes specifically reactive with target cells containing the polyproteins. The determination of the specific antigen determinants of the polyprotein that are immunologically active at the cell surface will await the development of monoclonal antibodies reactive with these specific determinants. Stable hybridoma cell lines have been prepared by polyethylene-glycol fusion of both the Y-3 and the NS-1 myeloma cell lines with immune lymphocytes obtained from hyperimmunized rats. The immunoglobulin products of these hybridomas are now being characterized and evaluation of the most optimal methods of producing reactive hybridomas are in progress.

Significance to Biomedical Research and the Program of the Institute:

The production of a library of monoclonal antibodies reactive with single antigenic determinants of these viral and cellular encoded transforming proteins will make possible the search for these proteins in, and a determination of, their role in spontaneous and chemically induced tumors.

Proposed Course:

Immune cells from regressor, progressor and hyperimmunized rats will be examined by cell fusion and hybridoma production for production of monospecific antibodies. The immunogens of current interest include FeSV and AbLV transformed nonproducer cell lines. Immunoglobulin production from such hybrids will be examined for reactivity with viral and cellular encoded transforming proteins using immunoprecipitation and gel electrophoresis techniques.

Publications:

Kende, M., Veronese, F., Hill, R., Dinowitz, M., and Kelloff, G.J.: Naturally occurring humoral immunity to endogenous xenotropic and amphotropic type C virus in the mouse. Int. J. Cancer 27: 235-242, 1981.

Kende, M., Veronese, F., Hill, R., Stephenson, J.R., and Kelloff, G.J.: Natural killer cell activity and humoral blocking activity to endogenous type C viruses of the mouse: Interaction of these two immune responses in the aging mouse and its implications for natural tumorigenesis. Cancer Res., in press.

*U.S. GOVERNMENT PRINTING OFFICE: 1981-341-132/3666--1-3